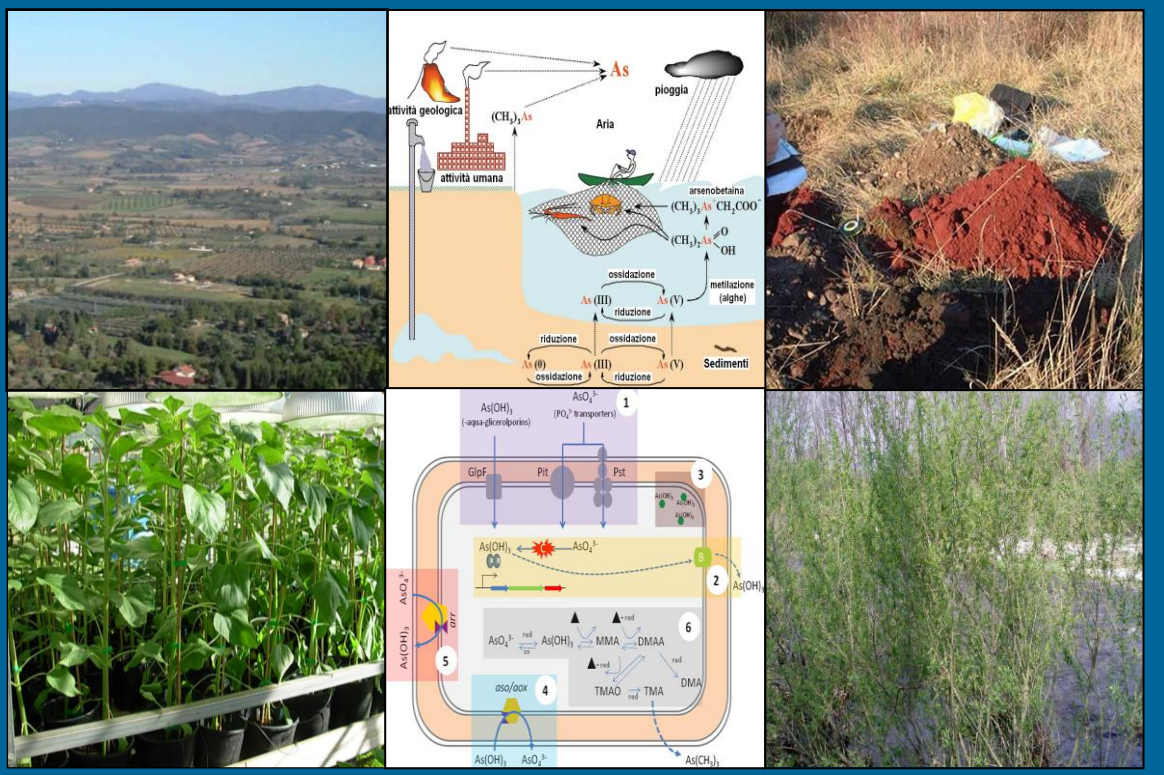
Microbial arsenic transformations in contaminated soils

ANNA CORSINI



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MICROBIAL ARSENIC TRANSFORMATIONS IN CONTAMINATED SOILS

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Ph.D. Thesis

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Summary

The work presented in this thesis explores the potential role of bacteria in the arsenic cycle an agricultural soil from Scarlino (Tuscany), and a soil from Torviscosa (Fiuli), pyrite cinders contaminated. Both soils contained high levels of total arsenic. The soil of Scarlino contained 250 mg kg^{-1}, mainly associated to iron oxides (70% of the total), while the labile forms were 10% of the total. Total As in the soil of Torviscosa was 446 mg kg^{-1}, mainly present in the residual phase (50% of the total), while the labile forms represented 15% of the total.

This thesis started to investigate the presence of rhizobacteria associated to *Cirsium arvense* (L.), a wild plant present in the soil of Scarlino, with the aim to isolate and characterize As-resistant bacteria with potential plant growth promoting characteristics and potentially involved in As transformations. FISH analysis of the rhizobacterial community of *C. arvense* revealed the presence mainly of *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*. Several isolates mainly belonged to *Bacillus*, *Achromobacter*, *Brevundimonas*, *Microbacterium*, and *Ochrobactrum* genera and resulted highly resistant to As(III) and As(V). In most of these strains *ArsC*, *ArsB* and *ACR3* genes, peculiar to the ars operon of the arsenic detoxification system have been detected. The ArsB genotype predominated over the ACR3, highlighting that the *ArsB* gene family is extensive; and the ArsB-type efflux pumps seemed to be predominant in *Firmicutes* and in *Betaproteobacteria*, and the ACR3-type in *Alphaproteobacteria*. Several isolates reduced As(V) and oxidized As(III). In particular, *Ancylobacter dichloromethanicum* strain As3-1b was able to transform both forms of As. Moreover, this strain was able to oxidize As(III) also under chemoautotrophic conditions.

Numerous As-resistant isolates possessed plant growth-promoting characteristics, being able to produce siderophores, IAA, and ACC deaminase. For this reason, these isolates could potentially support plant growth in As-polluted soil and reduce stress symptoms.

From a greenhouse experiment conducted on the soil from Scarlino with sunflower plants bacterized with an As-resistant PGPR strain, it was evidenced a synergistic role of the PGPR strain and the sunflower plants in As uptake. Moreover, the As-resistant strain colonization of the rhizozphere of sunflower was monitored by quantifying *ACR3(2)* gene, coding for the As(III) efflux pump. The results of the quantification of this gene evidenced that the strain colonized the sunflower rhizosphere.

As second part of this thesis we have investigated the effect of the addition of a C source on the fate of As and of the microbial population in the soil from Torviscosa, when is flooded. Moreover as the soil contained Fe and Mn oxides, we have investigated also their solubilization. Our results have confirmed that the presence of a readily utilizable carbon source for microorganisms can enhance microbial As solubilization.

The two C sources (glucose and citrate) used in the experimental microcosms, differently influenced the solubilization of As and Fe, as well as the bacterial community.

Solubilization of As and Fe resulted differently affected either in term of amount released or in term of kinetics of solubilization. In fact, citrate promoted two-fold higher solubilization of As and Fe, compared to glucose. This can be due to the effect of citrate on microbial community and to the wide and complex interactions among citrate and the dissolved ions occurring in a soil. In fact, citric acid, being a chelating agents, can modify the solubility of elements, such as iron and manganese. In addition, we found that citric acid influenced copper solubilization, thus increasing the ecotoxicological risk deriving from the flooding of multipolluted soils.

Different kinetics of solubilization of As and Fe under glucose and citrate were observed, confirming the complexity of the relationship between As and Fe release. By adding glucose to the soil, Fe solubilization was not concomitant to that of As, thus suggesting that desorption rather than dissolution was the main mechanism controlling release of arsenic from pyrite cinders. In glucose-amended soil, the

As(III) formation was governed by biological processes. The ability to reduce As(V) and the presence in soil DNA of genes belonging to the *ars* operon and of *arrA* genes confirmed that bacteria reduced As(V) by means of a detoxification systems and/or by a metabolic processes. In the presence of citrate, As(III) was the main species present in solution at oxic conditions, possibly due to the detoxifying activity of As(V) reducing bacteria; while As(V) was predominant at reducing condition. This was possibly due to the concomitant iron oxides dissolution that could have liberate As(V) occluded into Fe oxides.

Diverse bacterial populations were enriched by glucose or citrate. In fact, glucose stimulated populations of *Flavobacterium* and *Paenibacillus*, while citrate incremented those of *Bacillus*, *Pseudomonas*, *Clostridium*, and *Geobacter*. As-resistant bacteria were isolated either from glucose- or from citrate-amended microcosms, and with some of them we demonstrated their reducing capacities. Most strains possessed the *ars* genes, but not *arrA* genes. Quantification of *arsC* and *arrA* genes performed on soil microcosms revealed the constant presence of *arsC* in all microcosms, while *arrA* genes became evident only in glucose-amended microcosms, suggesting that the type of C differently stimulated the As-resistant microbial communities. The two functional genes *arsC* and *arrA* can be used a reliable biomarkers for detection of As-resistant bacteria responsible of As(V) reduction in contaminated soils. In particular, regarding *arrA* the method proved to be sensitive to detect a very low copy number (10 copy number g^{-1} soil).

In the final part of the thesis we have investigate the effect of milled alfalfa, a more complex C source, on As solubilization in the soil, under flooding condition. Beside microcosms experiment, a greenhouse experiment was also set up using the soil amended with milled alfalfa, and *Salix purpurea* (L.), in order to investigate As uptake and translocation in the plants. The solubilization of arsenic observed in submersion was mainly related to biological induced redox modification. However, the reductive conditions observed both in the microcosms and in the greenhouse experiment were not sufficient to allow Fe oxides dissolution and only a negligible release of Fe occurred. For this reason, it can be hypothesized that As mobilization

was due to reductive desorption from solid surfaces rather than to dissolution of Fe oxides, as in the case of glucose. Interestingly, solubilization of As in flooded soil during the greenhouse experiment was lower than that in microcosms soils, suggesting a direct or indirect effect of *S. purpurea* in the control of As level in the aqueous phase. From the results of As content in the leaves of willow, it possible to note that As accumulation capability of willows was low. Nevertheless, the plants demonstrated a good attitude to grow in a high contaminated soil subjected to flooding. For this reason, phytostabilization with willow could be a possible strategy in soils subjected to flooding.

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I

Introduction

1. Arsenic: a global concern

Though arsenic (As) is only the 20th most abundant element in the earth's crust, its toxicity is problematic in many parts of the world, because it contaminates soils, sediments and drinking water supplies. Figure 1 report the concentration and the distribution of As in different environmental media.

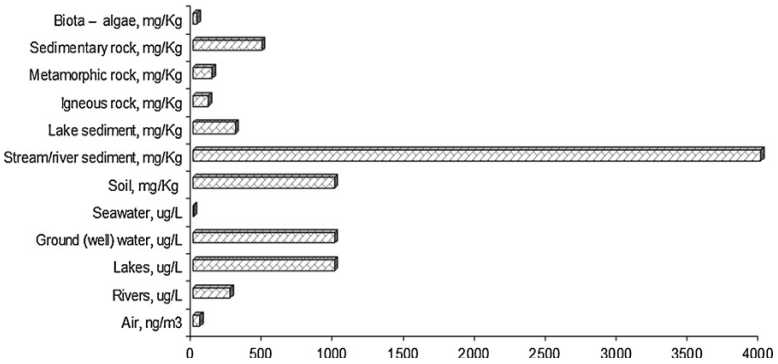

Figure 1. Arsenic concentrations in environmental media (data from U.S. Environmental Protection Agency, EPA, 2000).

Evidence of people suffering from chronic arsenic exposure has been recorded throughout history. Chemically, arsenic is very similar to phosphorus, and it can partially substitute for phosphorus in biochemical reactions.

Arsenic is toxic to plants and soil biota (Sadiq et al 1997); in humans, chronic exposure to inorganic arsenic can cause neurological damages, skin, lung, liver, bladder, and kidney cancers. Arsenite, As(III), is generally considered to be more acutely toxic than arsenate, As(V), and is also more potent than arsenate in chronic toxicity (Moore et al. 1997).

Total As concentration in soils can reach levels up to more than 1000 mg/kg (Mukhopadhyay et al., 2002) and in waters may reach concentrations as high as 5000 μg/L (Smedley and Kinniburgh, 2002). The World Health Organization recommends As guideline values of 10 μg/L in drinking water (WHO, 2001), but

As concentrations in many parts of the world (i.e. Bangladesh, Cambodia, Vietnam, West Bengal, and the United States) often exceed this limit. The current drinking water standard of set by WHO makes no differentiation between arsenite and arsenate. It is believed that arsenate is the major water-soluble species in groundwater, but there is increasing evidence that arsenite may be more prevalent than anticipated.

Both natural and human activities are responsible for the arsenic contamination in the world. Geochemical sources of As-contaminated soils include As-rich parent material as As easily substitutes for Si, Al or Fe in silicate minerals (Bhumbla and Keefer, 1994). Arsenic is also commonly associated with sulfides, e.g. in sulfidic ore deposits. Other natural sources of As include volcanic activities, low temperature volatilization and natural weathering of As-containing minerals (Wilkie and Hering, 1996).

The major anthropogenic sources which contribute to arsenic release in the environment are metal smelting industry, coal combustion, semiconductor industry, mine tailings and pigment production for paints and dyes. The use of arsenic in the chemical weapons has resulted in the contamination of several former military bases in Eastern Europe. Use of arsenic in medicine as chemotherapeutic agent for protozoan diseases, in agriculture as pesticides, animal feed additives particularly for poultry and in leather and wood treatments also contributes as additional sources of contamination (Welch et al., 2000).

2. Arsenic polluted sites

Over the past three decades, occurrence of high concentrations of arsenic in drinking-water and soils has been reported in several parts of the world. Before 2000, there were five major incidents of arsenic contamination in groundwater in Asian countries: Bangladesh, West Bengal, India, and sites in China (Mukherjee et al., 2006). In the last 10 years, arsenic-related groundwater problems have emerged in new sites in China, Cambodia, Thailand, Vietnam, DPR Korea, Pakistan, Argentina, Chile, United Stets and several parts in Europe.

In India, Bangladesh and Nepal the arsenic is of geological origin and occurs naturally in the aquifers, however in Thailand contamination of the groundwater has occurred as a result of mining activities. In Bangladesh alone, where As concentrations reach as high as 1000 μg/L, 50-60 million people are exposed to As-contaminated drinking water, and thousands of cases of arsenicosis are diagnosed each year.

In Argentina several wells, that are often the only drinking water resource available in rural settlements, are severely contaminated by arsenic. Arsenic occurrence in the aquifers of the area is of natural origin, related to volcanic ash, which is found dispersed in the sediments.

In the United States, the U.S. Geological Survey has developed maps that show where and to what extent arsenic occurs in ground water across the country. Widespread high concentrations were found in the West, the Midwest, and the Northeast of the country.

In several European countries As contamination of groundwaters and soils became evident in the last decades. Besides many hot spots, where high As concentrations in groundwater occur on a local scale, it is in the large regions of the Pannonian Basin (Hungary, Romania, Croatia and Serbia), the Kütahya Plain (Turkey), the region of Thessaloniki (Greece), Finland and Spain where high As concentrations

in groundwater constitute the most problematic cases in Europe. In the Pannonian Basin and in the region of Thessaloniki, As is released due to reductive dissolution of iron (oxy)hydroxides, while in the Kütahya Plain, As derives from geothermal activities. In Finland, the high As content in the wells is caused by natural sources and concentrations of arsenic are elevated in the fine fraction of till and fracture zones of the bedrock present in southern Finland (Jarva et al., 2008).

Cases of arsenic contamination caused by mining activities have been recorded in Germany, UK, France, Belgium and Hungary (Smedley and Kinniburgh, 2002), while incident causing As contamination occurred in Spain. In fact, in 1998, in Aznalcóllar a settling pond of a pyrite mine in broke open, spilling toxic tailings, containing also arsenic, into the basins of the Agrio and Guadiamar rivers (Simón et al., 1999).

In Italy, because of the complexity of its geological history and the wide variety of substratum rock types (Mantelli et al., 1999), the natural geochemical background is highly variable. Arsenic concentrations above the Italian threshold of 10 $\mu g\ L^{-1}$ have been recorded in several Regions, including Lazio, Tuscany, Lombardy, Veneto, and Sardinia. A study conducted on geographical distribution of bioavailable As in Italian agricultural soils highlighted that four provinces in the north-eastern Italy along with those of Cremona and Mantova (Lombardy) have the highest bioavailable As concentrations (Cubadda et al., 2010).

Beside natural sources, also anthropogenic activities are responsible for As contamination of Italian groundwater and soils. For example, in the 1960s in the Scarlino Plain (Tuscany) a big plant for the production of sulphuric acid from pyrite combustion was located. Since that time it has become evident that a large area around the industrial site is highly contaminated by arsenic compounds. This is not surprising since hematite cinders and other wastes of sulphuric acid production have been stored of in the ground without any control or protection.

Similarly, contaminated wastes derived from pyrite ore roasting for sulphur extraction were buried into soil (1938–1970s) adjacent to a chemical factory at Torviscosa (Udine, NE Italy), and consequently the soils resulted heavily contaminated by metals and arsenic.

3. Fate and mobility of arsenic in soils, sediments and waters

The fate and mobility of arsenic in the environment are mainly controlled by different processes that can affect the equilibrium of As precipitation and sorption reactions: (1) redox reactions; (2) adsorption and desorption; (3) competitive adsorption (ion exchange); (4) solid phase precipitation and dissolution; and (5) biological activity. Many factors such as redox potential (Eh), pH, dissolved organic carbon (DOC), and chemical speciation play a crucial role in these processes (Adriano, 2001; Islam et al., 2004; Bauer and Blodau, 2006; Al-Abed et al., 2007; Martin et al., 2007). Consequently, the occurrence, distribution and mobility of As are dependent on the interplay of these geochemical and biological factors (Cheng et al., 2009).

3.1. Redox reactions of arsenic and pH effects

Arsenic occurs in the environment in four oxidation states, which are denoted -3, 0, +3 and +5. Arsenite (H_3AsO_3) and arsenate ($HAsO_4^{2-}$) are the two inorganic species mainly present in natural waters and soils. Figure 2 shows the Eh-pH diagram for arsenic species. As(III) is generally found under reducing environments and neutral pH, while As(V) predominates under well-oxidized conditions (Ackermann et al., 2008). With decreasing pH, arsenic mobility tends to increase due to mineral dissolution, proton competition for surface binding sites, and increased surface potential (Pierce and Moore, 1982; Meng et al., 2001). On the other hand, an increase in pH can result in desorption of arsenic due to the lower stability of otherwise stable metal oxide-arsenic complexes (Masscheleyn et al., 1991; Raven et al., 1998).

Redox reactions can control aqueous arsenic concentrations by their effects on arsenic speciation and consequently As adsorption and desorption.

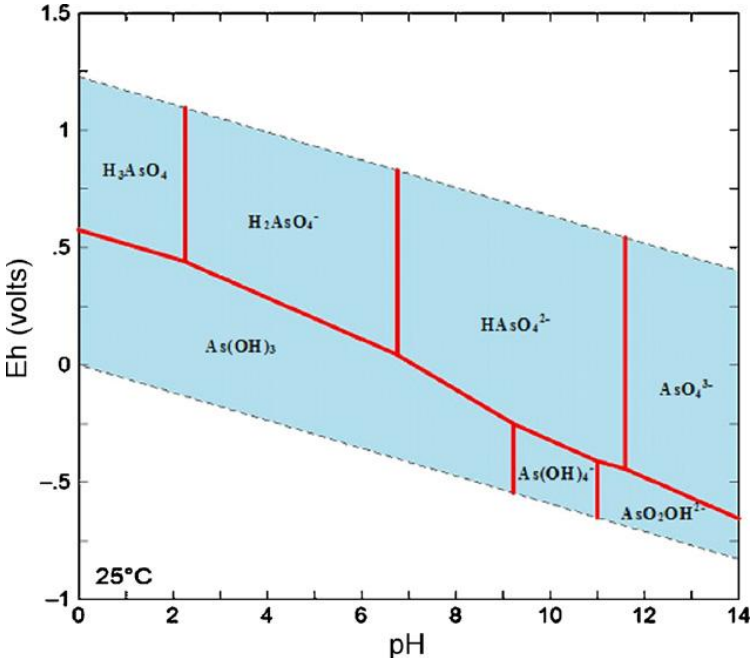

Figure 2. Eh-pH diagram for arsenic species.

Arsenic redox reactions can be predicted from thermodynamic properties of the redox couples that may be important for As(V)/As(III) redox reaction in soils and groundwater. Theoretically, the oxidized species of couples having positive Eh can oxidize the reduced species of couples having negative Eh at a given pH. Redox couple Mn^{3+}/Mn^{2+} have high oxidizing potential and are not stable in aqueous solution, and Fe^{3+}/Fe^{2+} cannot be stable in alkaline solution, either. Among the minerals, Fe(III) (hydro)oxide minerals can generally oxidize arsenite at pH below 4–5, but they are no longer capable of oxidizing arsenite at pH above 8.

Johnston and Singer (2007) found that Fe(II) could reduce arsenate in the presence of goethite, but not in homogeneous solution at near-neutral pH under anoxic conditions, while the oxidation of arsenite and Fe(II) depended heavily on pH buffer type and concentration under aerobic conditions. Manning and Goldberg

(1997) found that oxidation of arsenite to arsenate could be enhanced by heterogeneous oxidation on kaolinite and illite surfaces as well.

Mn(IV) (hydro)oxide minerals are not stable at pH below 4 to near 6, but they can serve as general oxidants for arsenite oxidation at pH above 6. Manganese oxides are highly redox-sensitive compounds and they play a distinctive role in the surface soil or near surface environments due to their narrow Eh–pH stability field. Arsenite can be readily oxidized by manganese oxides in lake water (Kuhn et al., 1994), sediments (Oscarson et al. 1980, 1981), and in aquifer materials (Amirbahman et al., 2006).

Concluding, manganese (hydro)oxides are responsible for arsenite oxidation under near-neutral to alkaline conditions, while iron (hydro)oxides are important oxidants under acidic conditions (Cheng et al., 2009).

Besides directly affecting the speciation of arsenic, redox reactions also control the stability of most sulphide and (hydro)oxide minerals, which are sources and sinks of arsenic species. Sulphide minerals are generally stable under reducing conditions, but they will be oxidized under oxidizing conditions, which will release the arsenic contained in them into the environment.

3.2. Adsorption and desorption of arsenic species

Adsorption of arsenic is a complex function of the interrelationship between properties of the solid surface, pH, the concentration of arsenic and competing ions, and arsenic speciation (Stollenwerk et al., 2002).

There are two general mechanisms for adsorption of arsenate and arsenite on mineral surface: non-specific adsorption and specific adsorption.

Non-specific adsorption, known as outer-sphere complex, involves the electrostatic attraction between a charged surface and an oppositely charged ion in solution as shown in Figure 3a. Specific adsorption, called inner-sphere complex, involves the formation of a coordinative complex between the mineral surface and ions in solution, even they do not posses oppositely charge (Figure 3b-d).

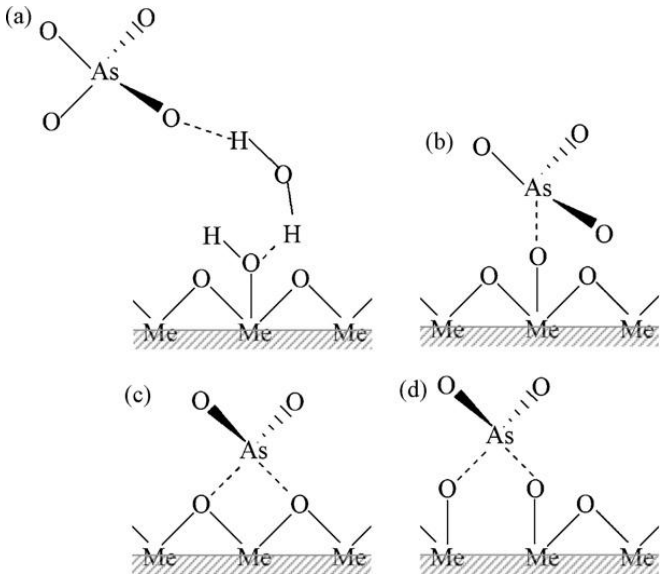

Figure 3. Schematic representation of configurations of arsenic molecules adsorbed on metal (hydro)oxide surfaces (Cheng et al., 2009): (a) outer-sphere surface complex; (b) mononuclear monodentate inner-sphere complex; (c) mononuclear bidentate inner-sphere complex; and (d) binuclear bidendate inner-sphere complex.

As(V) binds more strongly on positively charged surfaces like the (hydro)oxides of Fe, Mn, and Al (Adriano, 2001; Fitz and Wenzel, 2002), as compared to the As(III) species (Sadiq, 1997; Bissen and Frimmel, 2003). However, the binding mechanisms are dependent on the pH and redox potential of the environment. In fact, Eh and pH play a key role in controlling stabilities of (hydro)oxide minerals (e.g., iron, manganese and aluminum oxides or (hydro)oxides), and consequently the speciation and distribution of arsenic species between solid phases and aqueous phases. The strong adsorption of As(V) by Fe and Al (hydro)oxides at low pH and of As(III) at higher pH values has been observed in many experimental studies (Masscheleyn et al., 1991; Manning and Goldberg, 1997; Goldberg, 2002; Dixit and Hering, 2003).

Commonly occurring solutes with similar or higher charge densities, such as phosphate ($PO4^{3-}$), sulphate ($SO4^{2-}$), carbonate ($CO3^{2-}$) may compete for the same sorption sites on mineral surfaces affecting arsenate adsorption and desorption reactions. In particular, phosphate, which has similar chemical properties and behaviours as arsenate, strongly competes with arsenate for sorption sites on metal oxides surfaces (Sadiq, 1997; Liu et al., 2001; Violante and Pigna, 2002).

Besides the competition from inorganic anions, organic species may also interfere with arsenic sorption. The role of dissolved organic matter (DOM) and dissolved organic carbon (DOC) on the adsorption behaviour of arsenic species on a range of minerals and soils has been well documented (Xu et al., 1998; Grafe et al., 2001, 2002; Redman et al., 2002; Bauer and Blodau, 2006; Chen et al., 2008). Fulvic or humic acids form stable complexes with mineral surfaces, effectively blocking arsenic from adsorption on iron (hydro)oxides (Grafe et al., 2001).

It has also been demonstrated that DOM is able to chemically mobilize As from iron oxides, soils, aquifers, sediments and from arsenic-loaded materials, directly reducing As(V) as well as oxidizing As(III) (Redman et al., 2002; Bauer and Blodau, 2006; Mohapatra et al., 2007).

Recently, it was also proposed that microbially mediated Fe oxide reduction and subsequent dissolution, fuelled by the presence of labile DOM, resulted in the release of arsenic bound to sedimentary Fe oxides (McArthur et al., 2004; Wang and Mulligan, 2006).

Low molecular weight organic acids (e.g., acetate, lactate, oxalate, malate, succinate, gluconate, and citrate) are important exudates of plants and may influence the mobility and bioavailability of metals or metalloids. Organic acids with low molecular weight can affect As adsorption and desorption especially at acidic pH (Grafe et al., 2001; Zhang et al., 2005; Rong et al., 2009). Zhang et al. (2005) have demonstrated the significant influence of organic anions upon the release of arsenic from the soil, and the existence of a linear relationship between the released arsenic and the concentration of organic anions.

3.3. Precipitation and co-precipitation of arsenate and arsenite

In natural environments arsenate may form precipitates or co-precipitates with Al, Fe, Mn, and Ca (Sadiq et al., 1997) forming different arsenic-containing salts. Arsenite does not appear to directly precipitate in salt form with metal ions, but it precipitates in form of orpiment (As_2S_3) under reducing and sulfide rich environment. Iron and aluminium hydro(oxides) are of particular importance because of their wide occurrence and high arsenic sequestering capacity. In fact, Fe and Al are dissolved in solution at low pH, but precipitate out in form of hydro(oxides) in neutral to alkaline pH region. Oxidation of dissolved Fe^{2+} and precipitation of Fe(III) (hydr)oxides are established mechanisms for increasing As sorption, either by supplying new sorption sites for As or by co-precipitating Fe(III) with As (Choi et al., 2008). Precipitation and co-precipitation of arsenic with Fe and Al are practical and effective treatment processes used for removing arsenic from processed wastewaters, water treatment plants and from drinking waters (Hering et al., 1997; Roberts et al., 2004).

4. Microbial processes involved in As cycle and the sequestration of As

Microorganisms play a significant role in the biogeochemistry of arsenic in the environment by either direct or indirect mechanisms (Macur et al., 2001; Oremland and Stolz, 2005; Routh et al., 2007; Bachate et al., 2009). To counteract the deleterious effects of arsenic, bacteria have evolved several resistance strategies to transform arsenic, including arsenate reduction and active extrusion of arsenite from the cell, respiratory arsenate reduction, arsenite oxidation, as well as methylation into less toxic species (Figure 4) (Mukhopadhyay et al., 2002; Páez-Espino et al., 2009).

Microbial reduction of As(V) can occur through two mechanisms: a reductive intracellular mechanism of detoxification (Oremland and Stolz, 2003; Macur et al., 2004) or a dissimilatory reduction (respiration) (Oremland and Stolz, 2005). As(III) oxidation is carried out via detoxification reactions by heterotrophic bacteria (Ehrlich, 2002) or by respiration processes by chemoautotrophic bacteria (Santini et al., 2000; Oremland et al., 2002).

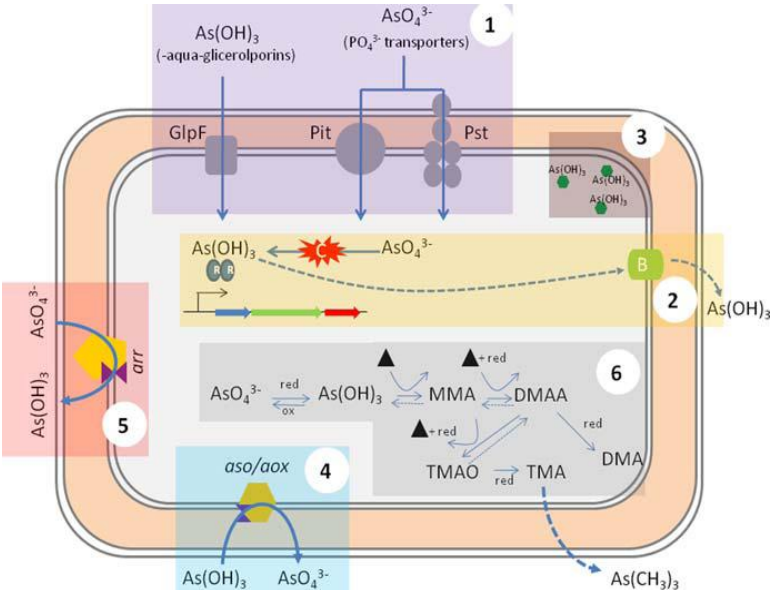

Figure 4. Diagram of the different microbial processes involved in arsenic biochemistry in the environment. (1) Arsenic enters the cells through the phosphate transporters (arsenate) or the aqua-glyceroporins (arsenite). (2) Once inside the cells, arsenate is reduced to arsenite by ArsC, which further extruded out of the cell by the specific pump ArsB. (3) Arsenite can also be detoxified by complexation with Cys-rich peptides. In addition, (4) arsenite can serve as electron donor by oxidation to arsenate. (5) Arsenate can be used as the ultimate electron acceptor during respiration. (6) Inorganic arsenic can also be transformed into organic species in a methylation cascade (Paèz-Espino et al., 2009).

4.1. Reductive intracellular detoxification mechanism

The detoxification mechanism usually occurs under oxic conditions and permits the cells to survive in a highly As contaminated environment without gaining energy. Arsenate ions enter the cells via phosphate transporters, due to structural homologies with phosphate ions (Figure 4). After reaching the cytoplasm, As(V) is reduced into As(III) before being excreted from the cell by a transmembrane protein. The genes encoding the arsenate detoxification mechanism (ars operons)

are widely distributed in bacteria and archaea and can be found on plasmids or chromosomes (Achour et al., 2007). They most commonly consist at minimum of either three (*arsRBC*) or five (*arsRDABC*) genes arranged in a single transcriptional unit: ArsB, an integral membrane protein that pumps As(III) out of the cell, often associated with an ATPase subunit, ArsA; ArsC, an As(V) reductase that converts arsenate to arsenite prior to efflux; ArsR, a trans-acting repressor involved in the basal regulation of the ars operon; and ArsD, a second repressor controlling the upper levels of ars genes expression (Achour et al., 2007). Two unrelated families of As(III) transport proteins responsible for As(III) extrusion have been described in bacteria (Mukhopadhyay, et al., 2002; Achour et al., 2007; Cai et al., 2009): the so-called ArsB protein and ACR3p arsenite carrier gene family.

The well-characterized ArsB family includes membrane proteins of *Escherichia coli* plasmid R773 and *Staphylococcus aureus* plasmid pI258, and it is prevalent in *Firmicutes* and *Gamma-proteobacteria*. ArsB has been extensively characterized and is a 45 kilodaltons, inner membrane protein with 12 transmembrane helices (Rosen, 1999). The ArsB functions as a uniporter using the membrane potential to extrude arsenite. When associated with ArsA, the permease is converted to a more efficient ATP-driven arsenite pump that provides enhanced arsenite resistance. In addition to arsenite, proteins of the ArsB family actively expel antimonite oxyanions (Meng et al., 2004).

Much less is known about the second family of arsenite carriers, Acr3p, that is mostly present in *Actinobacteria* and *Alphaproteobacteria* (Achour et al., 2007). Members of Acr3p transporters showed a function similar to ArsB, but the two proteins have no significant sequence similarity. Even though Acr3p is much less characterized, it has been reported to be present in more phylogenetically distant species than ArsB (Achour et al., 2007; Cai et al., 2009). Acr3p could be divided

into two subfamilies, Acr3(1)p and Acr3(2)p, based on their phylogenetic dissimilarities (Achour et al., 2007).

ArsC arsenate reductase is a small (13–16 kilodaltons) protein that is found in the cytoplasm of the microbial cell (Figure 5.A) and it's believed to be widespread among many groups of microorganisms. Although different studies report a common origin for the *arsC* genes (Butcher et al., 2000; Saltikov and Olson, 2002; Jackson and Dugas, 2003), Mukhopadhyay et al. (2002) suggests the existence of three distinct classes of arsenate reductases, which have developed similar mechanisms through convergent evolution. Two of these classes are bacterial and the third is the Arr2 gene of *Saccharomyces cerevisiae*. The two bacterial protein families differ in their structures, reduction mechanisms and the location of their catalytic cysteine residues (Páez-Espino et al., 2009). On one hand, there is the thioredoxin-coupled arsenate reductase class that includes the arsC proteine borne by *S. aureus* plasmid pI258, *Bacillus subtilis* and *Pseudomonas putida*; the second type of reductases, that includes *E.Coli* plasmid R773, uses glutaredoxin/glutathione in the corresponding biochemical reduction.

4.2. Arsenate dissimilatory reduction

Apparently, arsenate respiration occurs in a large variety of environments ranging from freshwater sediments, hypersaline lake waters, hot springs, deep-sea hydrothermal vents, gold mines, and arsenic-treated wood to bovine rumen fluid, and the termite hindgut (Laverman et al., 1995; Newman et al., 1997; Blum et al., 1998; Stolz et al., 1999; Gihring and Banfield, 2001; Niggemyer et al., 2001; Herbel et al., 2002; Saltikov et al., 2003; Takai et al., 2003; Santini et al., 2004). The first arsenic-respiring strain discovered was MIT-13 strain, a microorganism displaying the shape of a vibrio which was named *Geospirillum arsenophilus* (Lovley and Coates 1997). Lactate disappearance in the culture medium was observed to be proportional to the conversion of As(V) to As(III). To date, the

number of As(V) respiring bacteria has grown considerably. The isolation of numerous phylogenetically diverse strains of As(V)-respiring bacteria suggests that they are widely spread throughout the whole bacteria domain (Oremland and Stolz, 2005; Silver and Phung, 2005). An obligate anaerobic hyperthermophilic arsenic-respiring bacteria, *Pyrobaculum arsenaticum* DSM13514, was isolated from a hot spring at the Pisciarelli solfatara, Naples, Italy (Huber et al., 2000). Other extremophiles capable of arsenic respiration are *Bacillus arsenicoselenatis* and *B. selenitireducens* isolated from the anoxic muds of Mono Lake (California) an alkaline, hypersaline, arsenic-rich water body (Blum et al., 1998). Other bacteria with respiratory arsenate reductase activity, recently described, are *Chrysiogenes arsenatis* (Macy et al., 1996), *Desulfotomaculum auripigmentum* (Newman et al. 1997) (now reclassified as *Desulfosporosinus auripigmenti* corrig., comb. nov. by Stackebrandt et al., 2003), *Sulfurospirillum barnessi* and *S. arsenophilum* (Stolz et al. 1999), *S. barnesii* (Malasarn et al. 2004), *Desulfuroporosinus* sp. strain Y5 (Perez-Jimenez et al. 2005), *Wollinella succinogenes*, *Clostridium* sp. strain OhILAs, *Alkaliphilus metalliredigenes* (Stolz et al. 2006), *A. oremlandii* (Fisher et al. 2008), and *Shewanella* sp. strain ANA-3 (Malasarn et al. 2008).

Anaerobic microorganisms utilize As(V) as a terminal electron acceptor in dissimilatory arsenate respiration while oxidizing various organic (e.g. lactate, acetate, formate and aromatic compounds) or inorganic (hydrogen and sulphide) electron donors (Stolz and Oremland, 1999; Hollibaugh et al., 2002), resulting in cell growth (Figure 5b).

For example, while *C. arsenatis* can grow only in the presence of both arsenate and specific electron donors such as acetate, pyruvate, L- and D-lactate, fumarate, succinate, and malate (Macy et al 1996), *S. arsenophilum* can reduce As(V) using a variety of electron donors including lactate, acetate, hydrogen, CO_2 and sulphide (Oremland and Stolz, 2005). *Desulfosporosinus* sp. strain Y5, isolated from a multi-polluted sediment of Onondaga Lake (NY) is capable of using arsenate while

oxidizing various aromatic compounds (Pérez-Jiménez et al., 2005). Lear et al. (2007) reported that the introduction of a proxy for organic matter (13C-labeled acetate) in As-contaminated sediments from a Cambodian aquifer stimulated As(V) reduction. This was accompanied by an increase in the proportion of bacteria closely related to the dissimilatory As(V)-reducing bacteria *Sulfurospirillum* sp. strain NP-4 and *D. auripigmentum*.

Although the ability to respire As(V) is spread across several phylogenetic groups, the mechanism of As(V) reduction in these organisms seems to be conserved. Arsenate reduction is carried out by the respiratory arsenate reductase, a membrane–bound enzyme encoded in the *arr* operon which always includes the *arrA* and *B* genes in its genomic configuration (Páez-Espino et al., 2009). The first respiratory arsenate reductase, a periplasmatic heterodimer protein (87- and 29-kDa subunits) of the dimethyl sulfoxide family of mononuclear molybdenum-containing enzymes, was purified and characterized for *C. arsenatis* (Krafft and Macy, 1998). More recently, the respiratory arsenate reductase from the Gram-positive, haloalkaliphile, *B. selenitireducens* strain MLS10 was purified and characterized (Afkar et al., 2003); moreover, Malasarn et al. (2008) characterized the expression and activity of the *Shewanella* sp. strain ANA-3 arsenate respiratory reductase.

4.3. Arsenite oxidation

Numerous bacteria isolated from soils, mine tailings, river sediments and geothermal springs have been shown to oxidize As(III) (Santini et al., 2000; 2004; Oremland et al., 2002; Oremland and Stolz, 2003; Macur et al., 2004; Salmassi et al., 2006). As(III)-oxidizing bacteria are distributed among different genera including *Pseudomonas* (Turner, 1954), *Alcaligenes* (Anderson et al., 2002), *Thermus* (Gihring et al., 2001), *Thiomonas* (Duquesne et al., 2007), *Agrobacterium* (Kashyap et al., 2006), and *Herminiimonas* (Muller et al., 2006).

Microbial oxidation of As(III) is carried out by bacteria either heterotrophically or chemoautotrophically.

Heterotrophic As(III) oxidation has been extensively studied and is generally considered to be a detoxification reaction, which is catalysed by a periplasmatic enzyme that converts As(III) to As(V) on the cell's outer membrane (Figure 5.C) (Ellis et al., 2001; Anderson et al., 2002; Silver and Phung, 2005). The arsenite oxidase genes characterized for the heterotrophic As(III) oxidizers *Alcaligenes faecalis* (*asoAB* genes) and *Centibacterium arsenoxidans* (*axoAB* genes) show a 73% and 62% amino acid sequence homology (Silver and Phung, 2005). This enzyme contains two-subunits encoded by the genes *aoxA/aroB/asoB* (small Fe-S Rieske subunit) (Kashyap et al., 2006) and *aoxB/aroA/asoA* (large Mo-protein subunit) (Muller et al., 2006).

Unlike the heterotrophs, the arsenite-oxidizing chemoautotrophic microorganisms use the energy and reducing power from As(III) oxidation for CO_2 fixation and cell growth under both aerobic (Santini et al., 2000; Duquesne et al., 2008; Garcia-Dominguez et al., 2008) and nitrate-reducing (Oremland et al., 2002) conditions. Arsenite oxidation is a thermodynamically exergonic reaction ($\Delta G^{o'} = -256$ kJ/reaction) and can provide sufficient energy for As(III)-oxidizing chemolithotrophs (Santini et al., 2000). It has recently been reported that the genes (*aroAB*) involved in autotrophic As(III) oxidation by strain *Rhizobium* sp. NT-26 are induced by the presence of As(III), and the amino acid sequence is approximately 50% homologous to the *asoAB* and *axoAB* genes (Santini and vanden Hoven, 2004; Silver and Phung, 2005). Although the oxidase for the autotrophic As(III) oxidizers is similar to the heterotrophic oxidases, the difference in activity and lower sequence homology suggests that the *aroAB* genes may comprise a separate group.

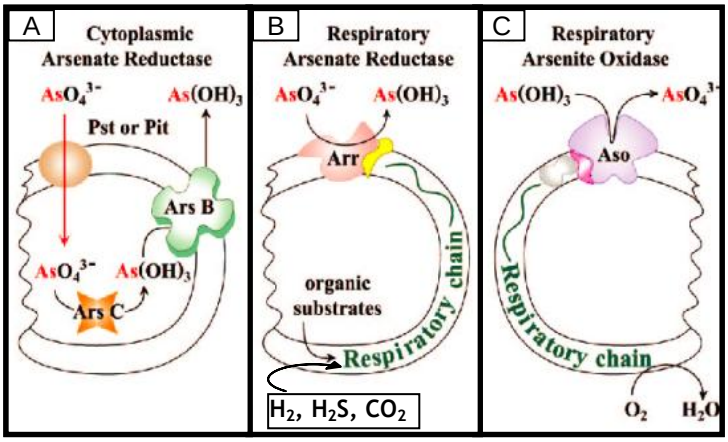

Figure 5. Cellular locations and functions of cytoplasmatic arsenate reducatese (A), respiratory arsenate reductase (B), and respiratory arsenite oxidase (C) (from Silver and Phung, 2005).

5. Decontamination processes

Several treatment technologies have been applied for the removal of arsenic from contaminated waters and soils. Processes commonly used for treating arsenic-contaminated waters are filtration, coagulation, ion exchange and inverse osmosis. At the first stage, most of these methods involved the oxidation of As(III) present into As(V) by adding powerful oxidants such as potassium permanganate, hydrogen peroxide or ozone. Conventional cleanup technologies applied to contaminated soils are soil removal, soil washing and physical stabilization. Current available cleanup methods are often high-priced, energy consuming, and in some cases they can be disruptive for the environment.

Biological and microbiological methods represents therefore economically viable and environmentally friendly alternatives for decontamination of waters and soils.

The application of biological processes for the oxidation of dissolved Fe and Mn have been proposed as an efficient treatment technology for the simultaneous removal of arsenic from waters (Katsoyiannis and Zouboulis, 2004; Tani et al., 2004). Katsoyiannis and Zouboulis (2004), for example, found that iron-oxidizing microorganisms naturally present in the groundwaters were capable to remove both As(III) and As(V) from groundwaters to concentrations below the WHO limit. The main product of biological oxidation of iron is usually a mixture of poorly ordered iron oxides often containing significant amounts of organic matter (Hassan et al., 2009). Arsenic can directly be adsorbed on the biogenic iron oxides and subsequently precipitate, or, according to Seith and Jekel (1997), even As(III) oxidation by iron-oxidizing bacteria may occur.

Under anaerobic conditions, arsenic can also be removed from groundwater by sequestering into insoluble sulfides indirectly by the metabolic activity of sulfate-reducing bacteria, utilizing a range of organic substrates with SO_4^{2-} as the terminal electron acceptor (Leblanc et al., 1996; Fukushi et al., 2003).

Since many bacteria play a crucial role in the As cycle, it seemed logical to envisage using them actively in bio-treatments of As-contaminated waters. Several laboratory experiments as well as pilot-scale studies of bio-treatments adopt a two stages approach combining an efficient As(III)-oxidizing strain, a suitable immobilizing material and an As(V) adsorbent. The first step consists in a biological oxidation of As(III) and the second step in the removal of As(V) produced by an adsorption process. The biological oxidation of As(III) can be performed either by previously isolated arsenite-oxidizing bacteria or by a bacterial natural consortium. Lièvremont et al. (2003) proposed a two stages approach consisting of an efficient As(III) oxidation by *Herminiimonas arsenicoxydans* immobilized on chabazite followed by an adsorption process of As(V) on a low cost arsenate adsorbent. Mokashi and Paknikar (2002) adopted a similar approach utilizing *Microbacterium lacticum* immobilized on brick pieces to oxidize As(III) and a successive As(V) removal with zero valent iron. Recent researches showed that the bioremediation of As(III) contaminated effluent can be developed using fixed-bed upflow reactor systems inoculated with an autotrophic As(III)-oxidizing bacterial consortium and filled with volcanic basaltic ash as a bacterial growth support (Battaglia-Brunet et al. 2006).

Even though As(V) reduction appears to be deleterious, in some cases, i.e. when arsenic is strongly sequestered, the performances of As(V) reducing bacteria can be used, as in the study of Chung et al. (2006). In fact, the authors examined the reduction of As(V) to As(III) and the precipitation or complexation of As(III) with sulfide or adsorbed to iron solids in a H_2-based membrane biofilm reactor.

Bioremediation processes can be also applied as an environment-harmless alternatives to As-contaminated soils. The term “bioremediation” describes several technologies and practices that take advantage of natural systems and processes to clean up pollution. The most commonly used soil bioremediation strategies are biostimulation, bioaugmentation and phytoremediation.

As the name implies, biostimulation involves some stimulation of the numbers and activities of natural populations, usually bacteria or fungi, so they can better break down pollutants into harmless products. Biostimulation is basically based on the assumption that a polluted environment already contains microorganisms that are capable of destroying or detoxifying pollutants. For this reason, provision of appropriate nutrient and suitable environmental conditions should allow natural pollutant clean up or stabilization to proceed. On the contrary, bioaugmentation is the practice of adding specialized microbes or their enzyme preparations to polluted matrices to accelerate transformation or stabilization of specific pollutants.
Phytoremediation techniques, such as phytoextraction and phytostabilization are defined as "the use of plants for containment, degradation or extraction of xenobiotics from water or soil substrates" (USEPA, 2000). The term phytoextraction refers to the use of plants for the cleanup of inorganics, combining high productivity with elevated uptake and translocation of pollutants to the harvestable biomass (Chaney et al., 2007). Being natural arsenic hyperaccumulators, ferns have been used extensively for bioremediation studies (Wei and Chen, 2006; Baldwin and Butcher, 2007). Other plant species have been used for phytoremediation of arsenic, including *Sorghum bicolour*, *Helianthus annuus* (Raab et al., 2005; Marchiol et al., 2007).
Phytostabilization focuses on long-term containment of the pollutant by reducing its mobility and dispersion in the environment, for example, by limiting the leaching from the soil. Species suitable for phytostabilization are often woody species (French et al., 2006; Tlustoš et al., 2007).
Recently the synergistic use of plants and bacteria was proposed as a new promising approach for remediation of metal contaminated soils (Glick, 2003). In fact, the use of plant growth-promoting bacteria as adjuncts in metal and arsenic phytoremediation can significantly facilitate the growth of plants in the presence of high (and otherwise inhibitory) levels of metals. Other possible benefits of

rhizobacteria on metal phytoremediation can be metal detoxification by immobilizing metals in the rhizosphere (Wu et al., 2006) and/or enhancement of metal accumulation in the plant biomass (Casiot et al., 2003; Zaidi et al., 2006).

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II

Overview

This PhD thesis focuses on the presence and the potential role of microorganisms in the arsenic cycle in two different Italian soils highly contaminated by arsenic: an agricultural soil from Scarlino (Tuscany), and a pyrite cinders contaminated soil from Torviscosa (Fiuli).

In Chapter 3, the rhizobacterial community of thistle *Cirsium arvense* (L.) Scopoli, a wild perennial plant growing in an As-contaminated soil of Scarlino (Tuscany), was examined. In particular, FISH analysis was used to obtain information about the community structure and the distribution of bacteria in rhizosphere fractions. Rhizobacteria have been isolated and screened for some plant growth promoting characteristics and for the presence of genes for As-resistance. Finally, As transformation capability of some isolates was determined.

Chapter 4 investigates the role of *Alcaligenes* sp. strain DhalL, an As-resistant strain with 1-amino-cyclopropane-1-carboxylic acid (ACC) deaminase activity, on As mobilization and translocation in *Helianthus annuus* (L.). Pot experiments were set up using the soil of Scarlino, sown with sunflower seeds inoculated with cell suspensions of *Alcaligenes* sp. DhalL. At different sampling time, As content in the aboveground part of plants was determined and the presence of the strain was monitored by quantification of the target gene *ACR3(2)*, coding for an As(III) efflux pump.

In Chapters 5 and 6 we have studied the temporal dynamics of the bacterial community and the effect of its activity on soil redox potential and on As, Fe and Mn solubilization in a submerged soil (Torviscosa) spiked with two different C sources, glucose and citrate.

The effect of the carbon source on the microbial populations associated with the As transformations was investigated using Denaturing Gradient Gel Electrophoresis (DGGE) analysis. The isolation of As(V)-reducing and As(III)-oxidizing aerobic heterotrophic bacteria was performed to confirm the role played by bacteria in arsenic reduction and solubilization from pyrite cinders.

Another part of my PhD thesis was focused on the set-up on new Real Time PCR protocols to quantify functional genes involved in the As cycle in the soil of Torviscosa. Optimization of the procedures have been performed under the supervision of Professor J. Sørensen and Professor M. Nicolaisen, during my staying as Visiting Scholar in the Genetics and Microbiology Laboratory at Life Science Faculty, University of Copenhagen. The results, reported in Chapter 7, are focused on the study of the dynamic changes in copy number of two genes potentially involved in arsenate reduction, *arsC*, and *arrA* in the soil of Torviscosa after the addition of glucose or citrate. Beside gene quantification, phylogenetic analysis of *arsC* and *arrA* genes was also performed in order to better understand the distribution and the diversity of the microorganisms involved in the As(V) reduction.

In Chapter 8 we have examined, in a greenhouse experiment, the effect of microbial decomposition of milled alfalfa, a more complex C source on As mobilization in this soil under flooded and not flooded conditions. Moreover, we have investigated arsenic uptake and translocation in *Salix purpurea* L.. The choice of this plant was due to its natural presence in the North of Italy and to the knowledge that it is resistant to high concentration of metals and arsenic.

Finally, Chapter 9 contains a brief summary of the key findings presented in this thesis and concluding remarks about the work.

III

Arsenic-resistant bacteria associated with roots of the wild *Cirsium arvense* (L.) plant from an arsenic polluted soil, and screening of potential plant growth-promoting characteristics

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Arsenic-resistant bacteria associated with roots of the wild *Cirsium arvense* (L.) plant from an arsenic polluted soil, and screening of potential plant growth-promoting characteristics

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Abstract

A rhizobacterial community, associated with the roots of wild thistle *Cirsium arvense* (L.) growing in an arsenic polluted soil, was studied by fluorescence in situ hybridization (FISH) analysis in conjunction with cultivation-based methods. In the bulk, rhizosphere, and rhizoplane fractions of the soil, the qualitative picture obtained by FISH analysis of the main phylogenetic bacterial groups was similar and was predominantly comprisedof *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*. The arsenic-resistant isolates belonged to 13 genera, the most abundant being those of *Bacillus*, *Achromobacter*, *Brevundimonas*, *Microbacterium*, and *Ochrobactrum*. Most bacteria grew in the presence of high arsenic concentrations (over 100 mM arsenate and 10 mM arsenite). Most strains possessed the *ArsC*, *ArsB* and *ACR3* genes homologous to arsenate reductase and to the two classes of arsenite efflux pumps, respectively, peculiar to the ars operon of the arsenic detoxification system. *ArsB* and *ACR3* were present simultaneously

in highly resistant strains. An inconsistency between 16S rRNA phylogenetic affiliations and the arsenate reductase sequences of the strains was observed, indicating possible horizontal transfer of arsenic resistance genes in the soil bacterial community. Several isolates were able to reduce arsenate and to oxidise arsenite. In particular, *Ancylobacter dichloromethanicum* strain As3-1b possessed both characteristics, and arsenite oxidation occurred in the strain also under chemoautotrophic conditions. Some rhizobacteria produced siderophores, indole acetic acid and 1-amino-cyclopropane-1-carboxylic acid deaminase, thus possessing potential plant growth-promoting traits.

Keywords: Rhizosphere bacterial community FISH Arsenic resistance genes Plant growth promoting traits Arsenic transformation

Abbreviations: FISH, fluorescence in situ hybridization; DAPI, 4,6-diamidine-2-phenylindole; ARDRA, amplified ribosomal DNA restriction analysis; ACC, 1-amino-cyclopropane-1-carboxylic acid; IAA, indole acetic acid; PCR, polymerase chain reaction; PGPR, plant growth-promoting rhizobacteria.

[$]Note: Nucleotide sequence data reported are available in the EMBL database under the accession numbers FN392622–FN392679.

Introduction

Arsenic (As) is ubiquitous in soils and its concentrations range from1 to 40mg $Askg^{-1}$ in uncontaminated soil, but can reach much higher levels in contaminated soils [36]. Arsenic mainly occurs in two inorganic forms, arsenite As(III) and arsenate As(V), both of which are highly toxic. Either form is found in the environment, although As(III) is generally found in reducing environments, while

As(V) predominates in well-oxidized conditions [3].

The metabolism of microorganisms influences the biogeochemical cycle of As, affecting both its speciation and toxicity. The tendency for As(III) to mobilize into the aqueous phase results from the differences in the sediment sorption characteristics of As(V) and As(III) [58]. Bacteria develop different As resistance mechanisms that can be divided into two basic categories consisting either of redox reactions that conserve the energy gained for cell growth or detoxification reactions through the ars operon genes [43,48,56]. The ars operon is well studied and its regulatory mechanism is better understood as compared to the redox reactions. A typical ars operon contains either three (arsRBC) or five (arsRDABC) genes that generally transcribe as a single unit [52]. ArsR is a repressor that binds the promoter region and regulates the ars operon. ArsB is a membrane-located transport protein that can pump As(III) out of cells using proton motive force. ArsC was shown to be a cytoplasmic As(V) reductase, whereas ArsA is an As(III)-activated ATPase [72].

Furthermore, ArsA and ArsB form an ArsA/ArsB complex that functions as a detoxification pump and thereby strongly enhances the efflux ability of As(III) [19]. ArsD regulates the ars operon as a weak secondary repressor [16,68]. The detoxification pathway occurs either aerobically or anaerobically and is not necessarily governed by prevailing redox conditions [30]. Arsenate respiration and detoxification are microbial processes that both contribute to As mobilisation in groundwater and soil [48]. As(V) is an oxyanion, which is chemically very close to the life-essential phosphate and can enter plant cells through existing phosphate transporters [40]. Consequently, the phosphate/As(V) uptake system plays a role in the influx of As(V) in plants.

Soil microorganisms affect As mobility and availability to the plant: they produce iron chelators and siderophores, reduce soil pH, and/or solubilise metal-phosphates

[1,34], thereby modifying the efficiency of the accumulation processes. Plants synthesise ‘‘stress’’ ethylene [42] from the precursor 1-amino-cyclopropane1-carboxylic acid (ACC) in response to biological and environmental stresses. Plant growth-promoting rhizobacteria (PGPR) that produce the enzyme ACC deaminase [25] cleave ACC and lower the level of ethylene, facilitating the formation of longer roots in plants growing in the presence of heavy metals [27]. PGPR-siderophore producers also help plants acquire sufficient iron for optimal growth in the presence of the heavy metals that hinder iron acquisition [29].

Molecular techniques allow the diversity of bacterial communities to be analysed in different habitats without cultivation. In particular, FISH enables the detection and identification of bacterial cells directly in their habitat [4] and thus provides a general picture of microbial community structure in plant-soil ecology studies [50,66].

Although microbial communities in As-polluted soils have been studied [5,49,62], little is known about the presence of As-resistant rhizobacteria associated with plants inhabiting As-polluted soils. Studies on As-resistant rhizobacteria provide new insights into bacterial diversity under unfavourable conditions for the exploitation of new isolates and information on As resistance genes in these habitats.

In this study, using independent- and culture-dependent techniques, the rhizobacterial community of thistle *Cirsium arvense* (L.) Scopoli, a wild perennial plant growing in an As-contaminated soil of Tuscany, was examined. The objectives were to: (i) obtain information about the community structure and the distribution of bacteria in rhizosphere fractions by FISH, (ii) analyse and characterise the rhizobacterial isolates and the presence of genes for As-resistance, and (iii) screen isolates for siderophore production, indole acetic acid (IAA), and ACC deaminase. Finally, the As transformation capability of some isolates was

determined, which probably contributes to the As cycle in the rhizospheres.

Materials and methods

Field site description and plant material

An intensive agricultural soil (La Botte site, Scarlino, GR, Tuscany, Italy) used for barley cropping at the time of sampling (June 2007) was studied. The site was heavily polluted by As, which was caused by either the natural presence of high concentrations of heavy metals or the extraction mining of pyrite associated with arsenopyrite, carried out in the last century.

C. arvense (L.) Scopoli (Asteraceae) was the most abundant wild plant species growing at the site. Thistles are sub-cosmopolite, sinanthropus species that usually grow on river and channel banks, in uncultivated grasslands and in landfills, and they become weeds in cultivated fields. A total of nine randomly chosen plants were collected at locations other than the cropped field. Only those plants that were surrounded by others of the same species as the nearest neighbours were extracted. The rhizospheric soil, highly impacted by the roots of the thistle, had a clay loam texture (containing 34% clay, 36% silt, 29% sand), and it had the following properties:pH (in water) 8.1,40 g kg^{-1} organic matter, available P 7.4 mg kg^{-1}, total N 2.01 g kg^{-1}, total $CaCO_3$ 67.3 g kg^{-1}, total As 250 mg kg^{-1}, labile As forms 25 mg kg^{-1}, and As bound to hydrous oxides of Fe and Al 181 mg kg^{-1}. The plants were extracted at points approximately 10 m apart, and the upper 20 cm of the plant root system with undisturbed soil around the roots was manually removed, placed in plastic bags to avoid moisture loss and delivered to the laboratory on the same day. Recovery of soil fractions from roots and microbiological analyses were performed the following day.

Preparation of rhizospheric soil fractions

The treatment of the root–soil system to obtain the three soil fractions (bulk, rhizosphere and rhizoplane) for separate analyses is described. The bulk soil fraction was obtained by carefully shaking the plants to remove non-adhering soil. The soil fractions collected from all samples were pooled together, homogeneously mixed, and then sieved (0.2 mm mesh width). The roots separated from the bulk soil fraction were grouped into three sets (about 10g roots wet weight), washed (1:10; w/v) in tetrasodium pyrophosphate (PP) (Sigma), pH 7.0, and vigorously shaken (180 rpm for 1h). The roots were then removed, the suspensions centrifuged (10,000 g, for 10 min at 4 °C), and the resulting pellets were defined as the rhizosphere fraction. Rhizoplane, the biofilm that closely adheres to root tissue, was obtained by ultrasonic treatment (UP100H Ultrasonic Processor, Hielscher, equipped with MS3 sonotrode, output control set at 50%, 3 times at 30 s each, and kept on ice) of roots suspended (1:5; w/v) in PBS (3 mM NaH_2PO_4, 7mM $NaHPO_4$, 130 mM NaCl, pH 7.2). The roots were then removed and the suspensions centrifuged (10,000 g for 10 min at 4 °C), and the resulting pellets (approximately 300 mg) were defined as the rhizoplane fraction. The dry weights of the fractions were calculated based on the moisture content (at 105 °C until constant weight was recorded). Viable counts of total heterotrophic and As-resistant bacteria were carried out both in the bulk and rhizosphere fractions, as described below. Aliquots of the three fractions were separately fixed for fluorescent microscopy analysis. Six replicates were added to 2 mL 3% paraformaldehyde in PBS, vortexed, and fixed for 12 h at 4°C in the horizontal position. Fixed suspensions were washed twice in filtered PBS, and the pellets, obtained by centrifugation (10,000 g for 10 min at 4°C), were suspended in 2 mL 50% (v/v) ethanol–PBS, and stored at -20 °C until use. To detect Gram-positive cells more accurately, six replicates (about 0.2 g wet

weight) of each fraction were fixed directly in 2 mL 50% (v/v) ethanol–PBS and stored at -20 °C.

Analysis of the rhizobacterial community

Fluorescence in situ hybridization (FISH) analysis

The rhizobacterial community was characterised by FISH analysis, as described by Bertaux et al. [9], using 16S rRNAtargeted oligonucleotide probes, labelled with fluorochrome Cy3 at the 5' end. The probe sequences employed and target bacterial groups are listed in Table 1. To obtain total counts of active bacteria, a combination of three EUB probes in equimolar mixture was used to detect most members of *Bacteria*, including *Planctomycetales* and *Verrucomicrobiales*. Defrosted samples were washed three times with PBS. Each pellet was then added to 2 mL Na_2EDTA 5 mM and 7 mg polyvinylpolypyrrolidone (Sigma), and shaken at 180 rpm for1 h to detach cells from soil particles. After settlement for 5min, 1 mL of supernatant was transferred to the top 1 mL of Nycodenz (Gentaur) with a density of 1.3 g mL^{-1}. The Nycodenz cell suspension gradient was centrifuged at 14,000 g for 30 min at 18 °C, and the upper 1800 mL of the gradient was collected for analysis. Two replicate aliquots of each cell suspension were added to PBS, and filtered onto 0.2 mm GTTP polycarbonate membranes (Millipore). The filters were successively dehydrated in 50%, 80%, and 96% ethanol, and then each filter was cut into four sections. Each section was aligned on silicon-coated slides and covered with 96 mL of hybridization buffer (900 mM NaCl, 20 mM Tris–HCl pH 8.0, 0.01% SDS) containing different percentages of formamide corresponding to the different probes (Table 1), and 4 mL of the labelled probe (50 ng mL^{-1}) (MWG-Biotech). The slides were placed in a moisture chamber and hybridised for 2 h at 46 °C. Then, at a temperature of 48 °C, filters were placed for 10 min in 1 mL of a washing solution (20 mM Tris–HCl, pH 8.0; 5mM EDTA, 0.01% SDS, pH 8.0)

containing NaCl in order to achieve the appropriate washing stringency (Table 1). Finally, the air dried filters were mounted on a microscope slide with antifading oil (Citifluor Ltd.), and examined under an epifluorescence microscope (Zeiss Axioskope) equipped with an HBO-50W mercury lamp and a Zeiss 15 filter set. Total microbial counts on the three fractions were also estimated by 4,6-diamidine-2-phenylindole (DAPI) staining [32], utilising the supernatants obtained after the Nycodenz gradient centrifugation, as described above. Two replicate aliquots (18 mL) of cell suspensions were added to PBS, mixed with the stain at a final concentration of 5 mg mL^{-1}, and stored in the dark for 15 min at room temperature. Samples were then filtered with black GTBP polycarbonate filters (Millipore). The air dried filters were mounted on a microscope slide, as previously described. The counts were determined with a fluorescence microscope (Zeiss Axioscope equipped with the Zeiss 01 set filter). The number of bacteria was determined by counting the cells in 20–30 microscopic fields using an eyepiece with a calibrated reticule. The counts were expressed as log cell numbers (g soil dry wt) $^{-1}$ for the bulk and rhizosphere fractions and as log cell numbers (g root dry wt)$^{-1}$ for the rhizoplane.

Viable counts

Three replicates of the bulk fraction (3 g each) and the rhizosphere fraction (0.2 g each) were suspended in PP solution (1:10; w/v) and shaken at 180 rpm for 1 h. Serial 10-fold dilutions in saline solution (0.9% NaCl) were prepared from bacterial suspensions. Heterotrophic bacteria were determined by plating aliquots (1 mL), in double sets of pour plates, of the various dilutions onto R2A (Difco), a medium suitable for the growth of diverse plant-associated bacteria. To study the As resistance level of the bacterial community, the number of heterotrophic As-resistant bacteria was determined by plating 1 mL of the various dilutions onto

R2A medium supplemented with either 15 mM of sodium arsenate, or 3 mM of sodium arsenite. Cycloheximide (0.1 g L^{-1}) was added to the media to inhibit fungal growth. Colony forming units (CFU) were counted after 10 days at 30 °C. The As resistance of the heterotrophs was expressed as percentage growth on R2A without the addition of As.

Isolation and identification of As-resistant strains

As-resistant bacteria were isolated from enrichment cultures in the presence of high concentrations of As to determine their response to As stress and to characterise their As resistance pattern. Rhizospheric soil samples (3 g) were used to inoculate flasks containing 50 mL each of a nutrient solution (hereafter referred to as BBM), which was modified from that described by Battaglia-Brunet et al. [7], and was made up as follows: Solution A – prepared in 500 mL demineralised water: KH_2PO_4 0.02 g; K_2HPO_4 0.02 g; NaCl 0.5 g; $(NH_4)2SO_4$ 0.2 g; trace element solution 1 mL. The pH of solution A was 6.5. Solution B – prepared in 500 mL demineralised water: $CaCl_2$ 0.1 g; $MgSO_4$ 0.1 g. Solutions A and B were sterilised separately by autoclaving. They were mixed together after cooling, and supplemented with 10 mL of a vitamin solution previously sterilised by filtration at 0.20 mm. The vitamin solution (L^{-1}) was: p-aminobenzoic acid 5mg; biotin 5mg; folic acid 2mg; pyridoxine–HCl 1mg; riboflavin 5mg; thiamine 5mg; nicotinic acid 5mg; panthotenic acid 5mg; and vitamin B12 0.1 mg. Just prior to use, the medium was supplemented with 6 g L^{-1} of sodium gluconate (BBMG) and with 15 mM of As(V) or 3 mM of As(III). The pH was adjusted to 8.0. The flasks were incubated by shaking (180 rpm) at 30 1C for 5 days. The 5 mL of each enriched culture were transferred into fresh medium and the cultures were incubated under the same conditions. To isolate As-resistant bacteria, appropriate dilutions of enrichment

cultures were plated onto R2A containing 15 mM As(V) or 3 mM As(III), and the plates were incubated at 30 °C for 10 days. After incubation, colonies with varying morphologies were selected from the plates and streaked for purity on the same medium. The strains were maintained in glycerol stocks at -70 °C. Prior to use, the strains were grown to mid-exponential phase in BBMG at 30 °C by shaking. Isolates were grouped based on amplified ribosomal DNA restriction analysis (ARDRA) using HhaI, HaeIII, and AluI restriction enzymes, and the 16S rRNA gene representative of isolates for each ARDRA group was sequenced. Strains were identified according to 16S rRNA gene sequence analysis.

Determination of As resistance and transformation by isolates

Resistance of each isolate to As(V) and As(III) was determined by growing them separately in 20 mL BBMG liquid medium to which As(V) (from 0 to 200 mM) or As(III) (from 0 to 50 mM) was added in increasing concentrations. Two vials of each concentration were inoculated with the appropriate cell suspension grown in BBMG without As in order to obtain a cell density of approximately 10^6 cells mL^{-1} ($OD_{620\ nm}$ of about 0.05). Their growth was evaluated by measuring the $OD_{620\ nm}$ after 5 days incubation at 30 °C. To test the ability of isolates to oxidise As(III) or reduce As(V), the strains were grown to mid-exponential phase in BBMG without As and they were then used to inoculate two vials containing 20 mL BBMG with 3 mM As(V) or 3 mM As(III) to obtain an initial $OD_{620\ nm}$ of about 0.05. Control flasks without inoculum were incubated to check abiotic transformation of As. Cells of strain As3-1b were grown in BBM medium supplemented with 0.5gL^{-1} $NaHCO_3$ (BBMC) to test whether the microorganism could oxidise As(III) autotrophically, since autotrophic species have been allocated to this genus [53]. Therefore, the strain, after three subsequent transplants in BBMC medium, was

used to inoculate two vials containing 20 mL of BBMC with 3 mM As(III). At different sampling times, aliquots of the growing cultures (2 mL) were removed in order to measure cell growth by measuring $OD_{620\ nm}$, and to determine the concentrations of As(V) and As(III) by spectrophotometric analysis, according to Dhar et al. [20]. Standard curves were prepared for concentrations of 0–100 mM for both As(V) and As(III).

Qualitative determination of plant-growth characteristics of isolates

1-amino-cyclopropane-1-carboxylic acid (ACC) deaminase activity

The enzymatic activity of ACC deaminase enables the use of ACC as the sole N source. The bacteria were cultured first in rich medium (TSB) and then transferred into DF medium containing 3.0 mM ACC, instead of $(NH_4)2SO_4$ as a source of N [18]. The cultures were incubatedona rotary shaker at 180 rpm for 48 h at 30 °C. Growth was positive when the cultures developed turbidity. The ability of a strain to utilize ACC was verified by inoculating the strain in control tubes containing DF medium without any N source, and incubating the tubes in the conditions described for 10 days. The absence of growth confirmed the utilization of ACC as a source of N.

IAA production

The bacteria were cultured for 4 days in flasks containing 20 mL DF medium supplemented with 0.5 mg mL^{-1} tryptophan. After incubation, 1 mL of the cell suspension was transferred into a tube and then mixed vigorously with 2 mL Salkowski's reagent (150 mL concentrated H_2SO_4, 250 mL distilled H_2O, 7.5 mL 0.5 M $FeCl_3.6H_2O$) [26] and allowed to stand at room temperature for 20 min, after

which the cell suspensions turned pink in colour.

Siderophore production

Siderophore secretion by strains was detected by the ‘‘universal’’ method of Schwyn and Neilands [55] using blue agar plates containing the dye Chrome azurol S (CAS) (Sigma-Aldrich). Orange halos around the colonies on blue agar were indicative of siderophore excretion.

Primer design for As resistance genes and polymerase chain reaction (PCR) conditions

A total of 50 bacterial gene sequences involved in As resistance were selected from GenBank and used for primer design. Primers P52f (5’-AGCCAAATGGCAGAAGC-3’) and P323r (5’-GCTGGRTCRTCAAATCCCCA-3’) were designed on the *ArsC* sequence of *Bacillus* spp. [5]. Primers P810f (5’-CACTSGCAARGTRMTCC-3’) and P1019r (5’-GTMGGCATGTTGTTCATG-3’) were designed on the consensus of *ArsB* sequences of *Alcaligenes faecalis* NCIB 8687 (acc. no. AY297781) and RS-19 (acc. no. DQ279766) strains. Achour et al. [2] reported primers darsB1F and darsB1R, dacr5F and dacr4R, and dacr1F and dacr4R that were used to amplify *ArsB, ACR3(2)* or *ACR3(1),* respectively. Degenerate nucleotide sites were indicated by standard ambiguity codes as follows: N = A, C, G, or T; R = A or G; V = A,C, or G and Y = C or T. PCR reactions were performed in a final volume of 25 mL containing: 10 ng total DNA, 0.2 mM of dNTPs, 1.75 mM $MgCl_2$, 0.4 mM of each primer, 2U Taq polymerase, and 1x PCR buffer. DNA amplification conditions for the P52f/P323r and P810f/P1019r pairs were: initial denaturation at 94 °C for 5min, 40 cycles at 94 °C for 45 s, at 55°C for 45 s, at 72°C for 30 s, and then a final extension step at 72 °C for 7 min. The same

conditions were applied to primer pair dacr1F/dacr4R, except that primer annealing was carried out at 50 1C. DNA amplification conditions for primer sets darsB1F/darsB1R and dacr1F/dacr1R were as described by Achour et al. [2]. All reagents were from Invitrogen. PCR reactions were performed on T-Gradient Biometra apparatus. The PCR products were checked on 2% agarose gel and visualized by ethidium bromide staining using the Gel Doc image analyser system.

Sequence analysis and phylogenetic tree construction

The gene sequences 16S rDNA, *ArsC*, *ArsB* and *ACR3(2)*, and *ACR3(1*) were compared to the entire GenBank nucleotide and amino acid databases using the BlastN and BlastX programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis of ArsC-and ACR3-deduced amino acid sequences were performed using MEGA version 4.0 software [59].

Chemicals

All chemicals were reagent grade. The stock solutions of As(III) and As(V) were prepared from $AsNaO_2$ (Fluka) and $Na_2HAsO_4.7H_2O$ (Fluka), respectively. All solutions were prepared with deionized water and were filter sterilised (0.2 mm, Millipore).

Results

Rhizobacterial community structure

The total bacterial counts determined by DAPI were 1–2 orders of magnitude higher than those obtained by FISH (Fig. 1 and Table 2), that accounted for 3%, 9% and 8% of DAPI counts, respectively, in the bulk, rhizosphere and rhizoplane

fractions. FISH analysis of the rhizobacterial community of *C. arvense* (L.) showed a similar qualitative picture at the level of the main phylogenetic microbial groups in the three fractions. Among the dominant bacteria, the Gram-negative *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* were the most abundant in all three fractions of the root–soil system. The three taxa together accounted for 49%, 70% and 93% of EUB338-positive bacteria in the bulk, rhizosphere, and rhizoplane fractions, respectively. The counts of Gram-positive, filamentous bacteria, spore-forming bacteria, as well as bacteria of the *Cytophaga-Flavobacterium* cluster were 1–2 orders of magnitude lower than the *Proteobacteria* and total counts. However, these values represented only a trend, since most of the numbers determined were close to the method detection limit. Rhizosphere counts were generally 1–2 orders of magnitude higher than the counts of the bulk fractions, confirming the rhizosphere effect. The counts of rhizoplane bacteria were not directly comparable to the other fractions because they were related to gram root dry weight (Table2). Comparing the bulk, rhizosphere, and rhizoplane fractions revealed some differences in the percentage distribution of the various taxa. The main differences were observed in the abundance of members of the phylum *Proteobacteria*, particularly in the rhizoplane that contained a higher percentage of *Gammaproteobacteria* (56% vs. 16% and 33%, respectively, in the bulk and rhizosphere), whereas *Alphaproteobacteria* were higher in the rhizosphere and rhizoplane (13% and 14%, respectively) than in the bulk (2%).

The abundances of culturable heterotrophs and As-resistant bacteria in the bulk and rhizosphere fractions are shown in Fig.2. As(V)-resistant bacteria were of the order of 10^7 CFU (g dry wt)$^{-1}$ of the bulk fraction and 10^8 CFU (g dry wt)$^{-1}$ of the rhizosphere fraction, and As(III)-resistant bacteria were of the order of 10^6 CFU (g dry wt)$^{-1}$ of the bulk and 10^7 CFU (g dry wt)$^{-1}$ of the rhizosphere fractions, accounting for 42–86% and 10–7% of the culturable counts, respectively. Numbers

of cultured heterotrophs were one order of magnitude lower than those determined with the combined EUB probes, confirming that the majority of soil microorganisms were ‘‘not cultured’’.

Identification of potential PGP As-resistant rhizobacteria

A total of 64 As-resistant bacteria were isolated from the enrichment cultures, that is, 46 were from the As(III)-enriched culture and 18 from the As(V)-enriched culture (Tables 3 and 4). The As resistance patterns of the isolates were varied, revealing high resistance to As(V) and moderate to high resistance to As(III). In stressed environments, rhizosphere bacteria with PGP characteristics could play an important role in plant growth. To identify potential PGP rhizobacteria, the 64 As-resistant isolates were qualitatively screened for ability to produce IAA, to utilize ACC as the sole N source, and to secrete siderophores into the growth medium. Tables 3 and 4 show that the 64 isolates possessed at least one PGP trait and, in particular, strains As3-5, As3-25, and As5-8 had all three traits.

The phylogenetic analyses indicated that the strains were allocated to 13 genera distributed among: *Alphaproteobacteria* (8 *Brevundimonas intermedia*,7 *Ochrobactrum tritici*,6 *Sinorhizobium*/ *Ensifer* spp., 2 *Bosea thiooxidans*,and 1 *Ancylobacter dichloromethanicum* strains), *Firmicutes* (16 *Bacillus* spp. strains), *Betaproteobacteria* (12 *Achromobacter* spp., and 1 *Bordetella* sp. strains), *Actinobacteria* (8 *Microbacterium*, 1 *Georgenia ferrireducens*, and 1 *Rhodococcus erythropolis* strains), and only one representative of the *Gammaproteobacteria* (*Pseudomonas veronii*). Some genera, such as *Bacillus*, *Ochrobactrum*, and *Ancylobacter* were peculiar to the As(III)-enriched culture and *Bosea*, *Rhodococcus* and *Georgenia* to the As(V)-enriched culture.

As transformation by isolates

All As(III)-resistant strains were tested for their ability to oxidise As(III), and at least one representative isolate for each genus of As(V)-resistant strains was tested for the ability to reduce As(V). In all, 16 isolates of As(III)-resistant bacteria (i.e. As3-3, -9, -17, -18, -20, -21, -22, -23, -36, -37, -1b, -2b, -10b, -5a, -9a, and -10a) oxidized As(III). It was noticeable that 10 As(III)-resistant strains (As3-3, -18, -36, -37, -1b, -2b, -10b, -5a, -9a, and -10a) completely oxidized 3mM As(III) in 24–48 h, while the other 6 isolates left from 50% to 86% of As(III) unoxidised even after a prolonged incubation period. *A. dichloromethanicum* As3-1b behaved as a facultative autotrophic As (III)-oxidizing bacterium (Figs. 3a and b). Moreover, it reduced As(V) to As(III) at a conversion level of 30% (Fig. 3c). Among the As(V)-resistant bacteria tested, only three strains (As5-2, -13, and -15) reduced 3mM As(V) between 25% and 50%.

Analysis of Ars genes in the isolates

ArsC genes of As(V) reductase and *ArsB* and *ACR3* genes of distinct types of the As(III) efflux pump were successfully amplified in 52 strains (Tables 3 and 4). The phylogenetic analysis of the deduced amino acid sequenze (Fig.4a) bifurcated ArsC into two distinct branches: the first was mostly made up of *Firmicutes* and *Actinobacteria* isolates, *Achromobacter* sp. strains As3-38 and As5-2, *A. dichloromethanicum* As3-1b, and *B. intermedia* As3-39. They carried similar ArsC sequences mostly homologous to the ArsC of *Bacillus* spp. (95–98% identical positions).

A. dichloromethanicum As3-1b possessed an ArsC moderately homologous (80%) to the ArsC of *Bacillus licheniformis* (Q65IV4). The second branch was composed of ArsC of *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*

isolates that carried ArsC moderately homologous (77–83% identical positions) to the As(V) reductase of *Herminiimonas arsenicoxydans* and to the tyrosine phosphatase of *Nitrosomonas europea*, respectively.

Two different types of As(III) efflux pumps were present in the isolates: 30 strains (mostly *Firmicutes* and *Betaproteobacteria*) carried an *ArsB*-type sequence and 22 strains (mostly *Alphaproteobacteria*) the *ACR3*-type. In particular, *ArsB* fragments were from moderately homologous (75%) to highly homologous (96%) to the As(III) efflux pump genes of *B. licheniformis* ATCC 14580 (CP000002), *Comamonas* sp. CNB-1 (DQ875599), *O. tritici* SCII24 (DQ490089), and *Acidithiobacillus caldus* (AY821803). ACR3(1) and *ACR3*(2) were present, respectively, in 7 and in 15 strains mostly belonging to *Alphaproteobacteria*. Phylogenetic analysis (Fig. 4b) clearly separated the *ACR3* sequences into the two types: *ACR3(1)* of the *Brevundimonas* strains and of *Sinorhizobium* sp. As5-8 were homologous to the As(III) efflux pump ACR3(1) of *Brevundimonas* sp. BAL3 (91%) and *Sinorhizobium medicae* (84%), respectively, whereas the *ACR3(2)* of the other As-resistant strains was grouped with the *ACR3*(2) (91–98% identity) of *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*. Clearly, *Sinorhizobium* sp. As5-9 carried the most divergent *ACR3(2)* sequence obtained (45% identity to *ACR3*(2) of *Ensifer* sp. TS16), and it formed a separate cluster on the phylogenetic tree.

ArsB and *ACR3(2)* were simultaneously present in three *Betaproteobacteria* strains, namely *Bordetella* sp.As3-3, *Achromobacter xylosoxidans* As3-10a, and *Achromobacter* sp. As5-10.

Discussion

The primary objective of this study was to identify the microorganisms that inhabit the rhizosphere of *C. arvense* growing in a soil with a long history of As pollution,

as an initial step towards identifying those organisms potentially involved in the aerobic As cycle.

FISH analysis was used to analyse the structure of the *C. arvense* rhizosphere-associated bacterial community and cultivation-based methods were used to characterize As-resistant bacteria. Our results showed a discrepancy between DAPI and EUB counts (accounting for o10%) in all the fractions of the root–soil system. This could be related both to the physiological state of the bacterial cells and to potential inaccuracies in our FISH protocol, applied by other authors [9] to study a sandy loam soil. However, large variations in EUB counts have been reported in different published reports. With regards to these results, a strong influence of the environmental chemical–physical characteristics, that affect both the composition of the microbial community at the phylogenetic level and the trophic state of the microbial soil populations, has been suggested [10]. Therefore, it could be hypothesized that the low EUB counts observed in our study could be related to the high As-pollution of the tested site. Generally, the reported variability in FISH effectiveness could probably be reduced by routine application of catalyzed reporter deposition (CARD-FISH) as an alternative method to conventional FISH for the detection of oligophilic bacteria. In fact, although this procedure is time consuming and more expensive, it has a high efficiency for specific staining and enumeration of soil microorganisms, independently of their ribosome content [21]. The predominance of Gram-negative bacteria in the root–soil system we studied was consistent with data in the literature, obtained by different methods, on the composition of other rhizosphere ecosystems [31]. However, there have been a higher percentage number of Gram-positive bacteria generally reported in the literature than those we determined in this study. This discrepancy could be due to different sampling methods employed for retrieving rhizosphere bacteria, as well as to the type of soil and plant analysed [8,37]. *Proteobacteria* seemed more tolerant

towards As than *Actinobacteria* and *Firmicutes*, whose numbers were greatly reduced, as also reported by Lorenz et al. [35]. It is noteworthy that the sum of the relative percentages of the phylogenetic groups explored compared to EUB338-positive bacteria differed in the three fractions examined, ranging from 49.9% in the bulk fraction to 70.6% and 96.7%, respectively, in the rhizosphere and rhizoplane fractions. This finding suggests that a large proportion of bacteria in the bulk fraction were most probably from other phylogenetic groups, such as *Acidobacteria*, *Verrucomicrobia*, and *Planctomycetes*, as reported for different soils [33,57,71] but not investigated in this study. A substantial proportion of the cultivable bacteria of bulk and rhizosphere fractions showed resistance to As(V) and As(III). In particular, As(V)-resistant bacteria represented a significant part of the cultured bacteria in the rhizosphere fraction (86%), thereby indicating that these populations had been under constant long-term metalloid stress and that the As in the soil could have exerted a selective pressure upon rhizosphere bacteria that would have developed a particular way to deal with the two As species.

The populations of cultivable rhizobacteria were analysed with respect to their As resistance and their PGP characteristics. The 64 aerobic As-resistant bacteria isolated showed a low degree of biodiversity at the genus level, being allocated to 13 genera. The abundance of *Bacillus* confirmed the strong resistance of this genus to conditions of stress [22]. The isolates displayed a high resistance towards both forms of As but to different extents, thereby suggesting that variation in As resistance could occur within bacterial populations, and perhaps representing an example of plasmid-borne As resistance. High As resistance might be due to the presence of a high expression level of the ars detoxification system [43] and to the presence of multiple copies of ars genes either on chromosomes or plasmids [47,60,69]. The simultaneous presence of *ArsB* and *ACR3* genes was associated with strains possessing medium to high As resistance. Arsenic resistance genes

ArsC, *ArsB*, or *ACR3* were retrieved from most isolates (81%). *ArsC* sequences of *Bacillus* sp. and isolates of *R. erythropolis* were conserved, being highly homologous to those of *Bacillaceae*. On the contrary, the *ArsC* of *Alphaproteobacteria* and *Betaproteobacteria* were more diverse and shared their homologies with different genera. The inconsistency in affiliation of the 16S RNA with strains having As(V) reductase sequence homologies could be due to horizontal transfer of As resistance genes in the bacterial community of highly contaminated soils, as found by Cai et al. [13] for *ArsB* sequences. The moderate homology of *ArsC* of *A. dichloromethanicum* As3-1b, especially to that of *B. licheniformis*, indicates a possible recent transfer of genetic material. In the isolates, *ArsC* was associated with an ArsB-type efflux pump and no *ArsC* was amplified in the strains carrying the ACR3-type transporter. Phylogenetic analysis of As-resistant bacteria by Muller et al. [44] showed the presence of two distinct classes of ArsC in the ArsB-type transporter operon and in the ACR3-type transporter operon, respectively. The primers P52F/ P323R designed would probably target only the first type of reductase. A variety of As(III) efflux pumps were present in the isolates, so much so, that the ArsB-type efflux pumps seemed to be the rule in *Firmicutes* and in *Betaproteobacteria*, and the ACR3type in *Alphaproteobacteria*. The presence of an ArsB-type in strain As3-1b is similar to the genetic organisation of the Ars resistance cluster genes in *O. tritici* SCII24T [11]. The ArsB genotype predominated over the ACR3 (30 vs. 22), suggesting that the *ArsB* gene family is extensive. The lack of success in amplification of As resistance genes in some strains might be due to the presence of sequences differing from the primers used, or to the existence of alternative mechanisms used to cope with As toxicity. Such mechanisms could include the production of volatile derivatives or the oxidation of As(III) to As(V). The latter explanation might be valid for *Microbacterium* sp. As 3-9, which oxidized As(III). In this work,

however, oxidase genes were not explored. In strains As5-4b and As5-8, the presence of the *ArsC* gene was not found compatible with the inability of these strains to reduce As(V). In these microorganisms, a system analogous to the *E. coli* phosphate-specific transporter could be present [15]. In isolate As5-13, As(V) resistance seemed to rely more on prevention of As(V) uptake. However, because a putative *ArsB* gene fragment was amplified, it is possible that As5-13 could reduce traces of As(V) when it enters the cells and then rapidly export As(III). In the conditions we studied here, a weak aerobic As(V) reduction was in fact observed. However, an alternative mechanism in this strain to reduce it to As(III) and efflux it cannot be ruled out. In the archaeon *Ferroplasma acidarmanus* F1, synthesis of heat-shock proteins HSP60 and HSP70, which are involved in protein refolding, was enhanced when the cells were exposed to As (III) [6]. This microorganism, although highly resistant to both As(V) and As(III), did not reduce As(V). Strains As5-4a and As5-16, highly resistant to As, were shown to contain putative *ArsC* genes, although they did not reduce As(V).This inability might be due to a variety of reasons, including point mutation in ArsC or separation of ArsC from its derepressible promoter. Furthermore, in As5-16, as well as in strain As5-15, which instead showed a weak As(V)reducing activity, a putative ArsC, clustering more tightly with tyrosine phosphatase rather than with As reductase, was found.

Rhizobacteria isolated with As(III)-oxidizing activity were more abundant than those with As(V)-reducing activity, contrasting with that found in the agricultural soils of Bangladesh [5]. This suggests that As oxidizing bacteria could play a possible protective role towards *Cirsium* plants. Arsenite is generally considered more toxic than As(V) for most plants. However, plants vary in their sensitivity or resistance to As [40]. Rhizobacteria encounter As(V) and As(III) in soil solutions before they enter the root, and oxidizing As(III) from soil solutions might help the plant to grow on As-contaminated soils and thus lower As(III) toxicity. The

presence of numerous As-resistant bacteria endowed with plant growth-promoting characteristics able to produce siderophores, IAA, and ACC deaminase, could potentially support plant growth in As-polluted soil and reduce stress symptoms. One or more of these mechanisms could be utilised by the plants at various times during their life cycle, as shown for many PGPRs [12,67,70]. Recently, a bacterial siderophore of mixed type containing catecholate and hydroxamate was found to remove As fractions – both the leachable and the bound – with a high degree of efficiency [45].

In our study, among As(III)-oxidizing bacteria, *A. dichloromethanicum* As3-1b oxidized As(III) using HCO_3^- as a carbon source, behaving as a facultative autotrophic, growing also on gluconate and other substrates (data not shown). To date, most of the chemoautotrophic As oxidizing-bacteria have been isolated from extreme environments such as gold and sulphur pyrite mine wastewater [54,64], thermal environments [24], and alkaline lakes [28]. The isolation of A. dichloromethanicum As3-1b from the rhizosphere of *Cirsium* augments the knowledge available on the broad distribution of autotrophic As-oxidizing bacteria present in contaminated soil [23]. The ability of *A. dichloromethanicum* As3-1b to perform both As(III) oxidation and As(V) reduction is a remarkable characteristic, as the strain could impact As mobility in aerobic environments. An accelerated conversion of As(III) to a more readily adsorbed species, As(V), might in fact attenuate transport of As(III) in the aqueous phase. However, how the strain deals with the two processes is still unknown and needs further work.

Papers published so far have considered the ability of bacteria to mobilize As in soil [14,61] and there are very few studies on the use of rhizobacteria in phytoremediation of As-contaminated soil. Rhizobacteria involved in mobilising insoluble nutrients by producing various organic acids might desorb As as well. An *Alcaligenes* sp. isolated from soil was found to be able to oxidise As(III)to As(V)

that plants could uptake via phosphate transporters [63].

Various microbial populations present in the root–soil system of *C. arvense* are able to withstand high As concentrations. Selecting microorganisms that are both metal-resistant and able to produce plant growth-promoting compounds could prove useful as inocula in re-vegetation and phyto-remediation processes.

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Table 1. Probes used and target bacterial groups

Probes	Sequence (5'-3')	Reference	Target organisms	*FA (%)	**NaCl (mM)
EUB338	GCTGCCTCCCGTAGGAGT	[4]	Bacteria	30	102
EUB338-II	GCAGCCACCCGTAGGTGT	[17]	*Planctomycetales*	30	102
EUB338-III	GCTGCCACCCGT AGGTGT	[17]	*Verrucomicrobiales*	30	102
NONEUB338	ACTCCTACGGGAGGCAGC	[65]	Negative control	30	102
BET42a	GCCTTCCCACTTCGT TT	[39]	*Betaproteobacteria*	35	70
GAM42a	GCCTTCCCACATCGT TT	[39]	*Gammaproteobacteria*	35	70
PSE227	AATCCGACCTAGGCTCATC	[66]	*Pseudomonas* spp.	30	102
ALF968	GGTAAGGTTCTGCGCGTT	[46]	*Alphaproteobacteria*	20	215
CF319a	TGGTCCGTGTCTCAGTAC	[38]	*Cytophaga-Flavobacterium* cluster of the CFB phylum	35	70
HGC69a	TATAGTTACCACCGCCGT	[51]	Gram-positive bacteria with high DNA G+C	25	149
LGC354b	CGGAAGATTCCCTACTGC	[41]	Gram-positive bacteria with low DNA G+C	35	70

Competitors were used for: BET42a (GCC TTC CCA CAT CGT TT), GAM42a (GCC TTC CCA CTT CGT TT) and HGC69a (TAT AGT TAC GGC CGC CGT) to improve in situ accessibility and specificity

*, % formamide (FA) in hybridization buffer;

** mM NaCl in washing solution

Table 2. Bacterial numbers obtained by DAPI and FISH analyses in the rhizoplane fractions of the root-soil system. Results are expressed as Log of cell numbers (g dry wt root)$^{-1}$. Values are means (n=6) ± S.E. Probes used are listed in Table 1.

Probes	Counts	*Eub338I, II, III %
DAPI	7.30 ± 0.12	
EUB338I, II, III	6.29 ± 0.19	
Bet42a	5.70 ± 0.12	24
Gam42a	6.03 ± 0.18	56
Pse227	5.95 ± 0.27	50
Alf968	5.36 ± 0.30	13
CF319a	3.99 ± 0.17	0.6
HGC69a	4.12 ± 0.20	0.9
LGC354b	4.22 ± 0.17	1.1

* Percentage of group-specific cell related to EUBI, II, III-positive cell counts.

Table 3. As (III)-resistant isolates enriched on BBMG with 3 mM arsenite, their As(V) and As(III) resistance levels, arsenic-resistance genes and PGP characteristics. *, percentages of homology to GeneBank relatives are reported for 16S rDNA sequences of representative strains¸[§], the resistance to As(V) and As(III) was evaluated by an increase in OD_{620} nm (from twofold to fivefold) of the inoculum. As(III) concentrations higher than 50 mM, and As(V) concentrations higher than 100 mM were not tested¸-, not detectable/ no production; [a], amplified with primers darsB1F/darsB1R; [b], amplified with primers P810f/P1019r

Isolates and closest relatives*	As resistance[§] mmol l^{-1}		PCR product					Phenotypic traits		
	As(III)	As(V)	*ArsC*	*ArsB*[a]	*ArsB*[b]	*ACR3(1)*	*ACR3(2)*	ACC-deaminase activity	IAA production	Siderophore production
As3-1b *Ancylobacter dichloromethanicum* 98% EU589386	3	100	+	-	+	-	-	+	-	-
As3-1 *Brevundimonas intermedia* 99% FJ609705	25	100	-	-	-	+	-	-	+	+
As3-2b *Achromobacter xylosoxidans* 99% AF439314	3	100	-	+	-	-	-	+	-	-
As3-2 *Bacillus* sp.	25	100	+	-	+	-		+	-	-
As3-3 *Bordetella* sp. 99% FJ598334	10	100	-	+	+	-	+	+	-	-
As3-4 *Bacillus* sp.	25	100	+	-	+	-	-	+	+	-
As3-5a *Ensifer adhaerens* 100% FJ609715	3	10	-	-	-	-	+	+	-	+
As3-5 *Microbacterium* sp. 98% FJ595885	25	100	-	-	-	-	-	+	+	+
As3-6 *Microbacterium* sp.	25	50	-	-	-	-	-	-	-	-
As3-7 *Ochrobactrum tritici*	25	25	-	-	-	-	+	-	+	-
As3-8 *Ochrobactrum tritici* 100% EU870448	25	100	-	-	-	-	+	-	+	-
As3-9a *Ensifer adhaerens*	3	100	-	-	-	-	+	+	-	+
As3-9 *Microbacterium* sp.	50	50	-	-	-	-	-	-	-	-
As3-10b *Achromobacter xylosoxidans*	3	50	-	+	-	-	-	+	-	-
As3-10a *Achromobacter xylosoxidans*	3	100	-	+	-	-	+	+	-	-
As3-10 *Bacillus* sp. 99% EU124558	50	100	+	-	+	-	-	-	+	-
As3-11 *Pseudomonas veronii* 99% FM162562	25	100	-	-	-	-	-	-	-	-
As3-12 *Bacillus* sp.	25	100	+	-	+	-	-	-	-	-
As3-13 *Bacillus* sp.	25	100	+	-	+	-	-	-	+	-
As3-14 *Bacillus* sp.	50	100	+	-	+	-	-	-	-	-
As3-15b *Ochrobactrum tritici*	25	50	-	-	-	-	+	-	+	+
As3-16 *Brevundimonas intermedia*	5	50	-	-	-	+	-	-	-	+
As3-17 *Bacillus* sp.	25	100	+	-	+	-	-	-	+	-
As3-18 *Bacillus* sp.	10	50	+	-	+	-	-	-	+	-
As3-19 *Brevundimonas intermedia*	25	100	-	-	-	+	-	-	+	+
As3-20 *Ochrobactrum tritici*	10	100	-	-	-		+	-	-	-
As3-21 *Bacillus* sp. 99% EU124558	25	100	+	-	+	-	-	-	-	-
As3-22 *Ochrobactrum tritici* 100% EU301689	25	50	-	-	-	-	+	+	+	-
As3-23 *Ochrobactrum tritici*	25	50	-	-	-	-	+	-	+	-
As3-24 *Microbacterium* sp.	25	50	-	-	-	-		-	-	+
As3-25 *Ochrobactrum tritici* 100% EU301689	25	100	-	-	-	-	+	+	+	+
As3-26 *Bacillus* sp.	50	100	+	-	+	-	-	-	+	-
As3-27 *Bacillus* sp.	25	50	+	-	+	-	-	-	-	-
As3-28 *Bacillus* sp.	25	50	+	-	+	-	-	-	-	-
As3-29 *Microbacterium* sp.	50	10	-	-	-	-	-	-	+	-
As3-30 *Bacillus* sp. 100% EU612335	50	100	+	-	+	-	-	-	+	-
As3-31 *Bacillus* sp.	25	100	+	-	+	-	-	+	-	-
As3-32 *Bacillus* sp.	25	100	+	-	+	-	-	-	-	-
As3-33 *Brevundimonas intermedia* 99% FJ609705	25	50	-	-	-	+	-	-	+	+
As3-34 *Bacillus* sp.	25	100	+	-	+	-	-	-	-	-
As3-35 *Microbacterium* sp. 99% FJ595885	25	50	-	-	-	-	-	+	-	-
As3-36 *Achromobacter* sp.	25	100	+	-	-	-	-	+	+	-
As3-37 *Brevundimonas intermedia* 99% FJ609705	25	100	-	-	-	+	-	+	+	-
As3-38 *Achromobacter* sp. 100% EU073119	25	10	+	+	-	-	-	-	-	+
As3-39 *Brevundimonas intermedia*	10	100	+	-	+	-	-	+	+	-
As3-40 *Brevundimonas intermedia*	25	10	-	-	-	-	-	+	+	-

Table 4. As(V)-resistant isolates enriched on BBMG with 15 mM arsenate, their As(V) and As(III) resistance levels, arsenic-resistance genes and PGP characteristics

Isolates and closest relatives*	As resistance[§] mmol l^{-1}		PCR product					Phenotypic traits		
	As(V)	As(III)	*ArsC*	*ArsB*[a]	*ArsB*[b]	*ACR3(1)*	*ACR3(2)*	ACC-deaminase activity	IAA production	Siderophore production
As5-1 *Achromobacter* sp.	200	10	-	-	+	-	-	-	-	-
As5-2 *Achromobacter* sp.	200	5	+	+	-	-	-	-	-	-
As5-3 *Sinorhizobium* sp. 99% AY505132	200	10	-	-	-	-	+	-	-	+
As5-4a *Rhodococcus erythropolis* 99% U81990	200	2	+	-	-	-	-	-	-	+
As5-4b *Bosea thiooxidans* 99% AJ250798	200	10	-	-	-	-	-	-	-	-
As5-5 *Bosea thiooxidans*	200	5	+	-	-	-	-	-	-	+
As5-6 *Sinorhizobium* sp.	200	2	-	-	-	-	+	-	-	+
As5-7 *Achromobacter* sp.	200	5	-	+	+	-	-	-	-	+
As5-8 *Sinorhizobium* sp.	200	10	-	-	-	+	+	+	+	+
As5-9 *Sinorhizobium* sp.	200	5	-	-	-	-	+	+	+	-
As5-10 *Achromobacter* sp. 99% EU073119	200	5	-	+	+	-	+	-	-	+
As5-11b *Microbacterium* sp.	200	2	-	-	-	-	-	-	-	+
As5-11a *Microbacterium* sp. 99% FJ595885	200	5	-	-	-	-	-	-	-	+
As5-12 *Georgenia ferrireducens* 99% EU095256	200	2	-	-	-	-	-	-	-	+
As5-13 *Achromobacter* sp.	200	10	-	+	-	-	-	-	-	-
As5-14 *Achromobacter* sp.	200	5	-	+	+	-	-	-	-	-
As5-15 *Achromobacter* sp.	200	5	+	-	+	-	-	-	-	-
As5-16 *Brevundimonas bacteroides* 97% AJ227782	200	5	+	-	-	+	-	-	-	-

*, percentages of homology to GeneBank relatives are reported for 16S rDNA sequences of representative strains

§, the resistance to As(V) and As(III) was evaluated by an increase in OD_{620} nm (from twofold to fivefold) of the inoculum. As(V) concentrations higher than 200 mM and As(III) concentrations higher than 10 mM were not tested

-, not detectable/ no production

[a], amplified with primers darsB1F/darsB1R

[b], amplified with primers P810f/P1019r

Fig. 1. Bacterial numbers obtained by DAPI and FISH analyses in bulk (□) and rhizosphere (■)fractions. Results are expressed as Log of cell numbers (g dry wt soil or root)$^{-1}$. Values are means (n=6) ± S.E. The percentage of group-specific cell counts within the EUB-positive cells is given above each bars. Probes used are listed in Table 1.

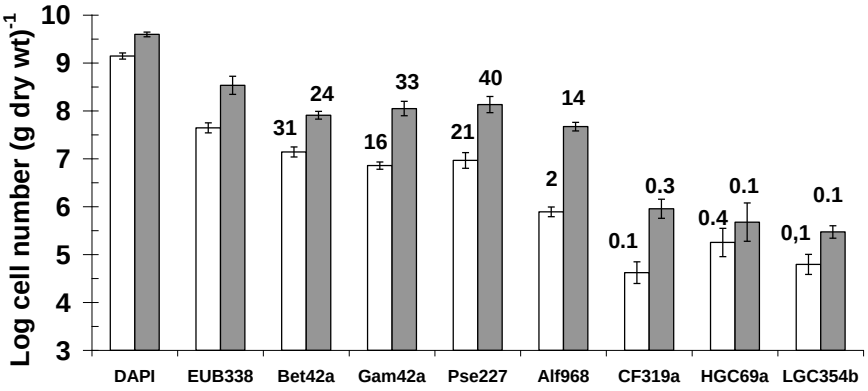


Fig. 2. Culturable counts in bulk (□) and rhizosphere (■) fractions. H, heterotrophic bacteria grown on R2A medium; As(V)-R, arsenate-resistant bacteria grown on R2A with 15 mM As(V); As(III)-R, arsenite-resistant bacteria grown on R2A with 3 mM As(III). Results are expressed as Log CFU (g dry wt soil)$^{-1}$ Values are means (n=3) ± S.E. The percentage of As(V) and As(III)-resistant bacteria within heterotrophic bacteria is given above each bars.

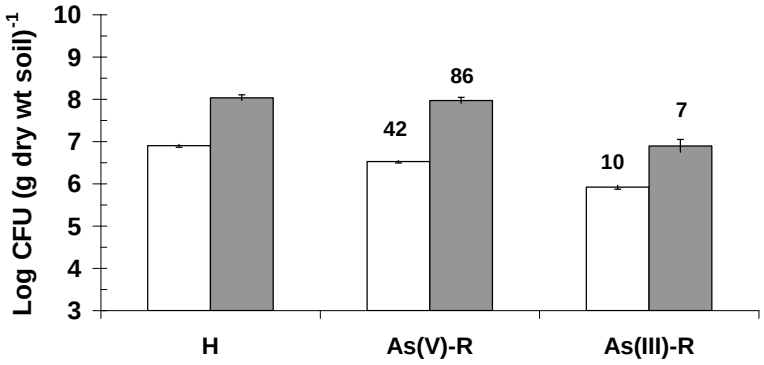


Fig. 3. Time course of bacterial growth as O.D. 620_{nm} (—•—) and As transformation rate by *Ancylobacter dichloromethanicum* As3-1b in BBMG (a, c) and BBMC medium (b): concentration of arsenite with (--▲--) and without cells (--Δ--); concentration of arsenate with (-·■-·) and without cells (-·□-·). Each value is the mean of two determinations.

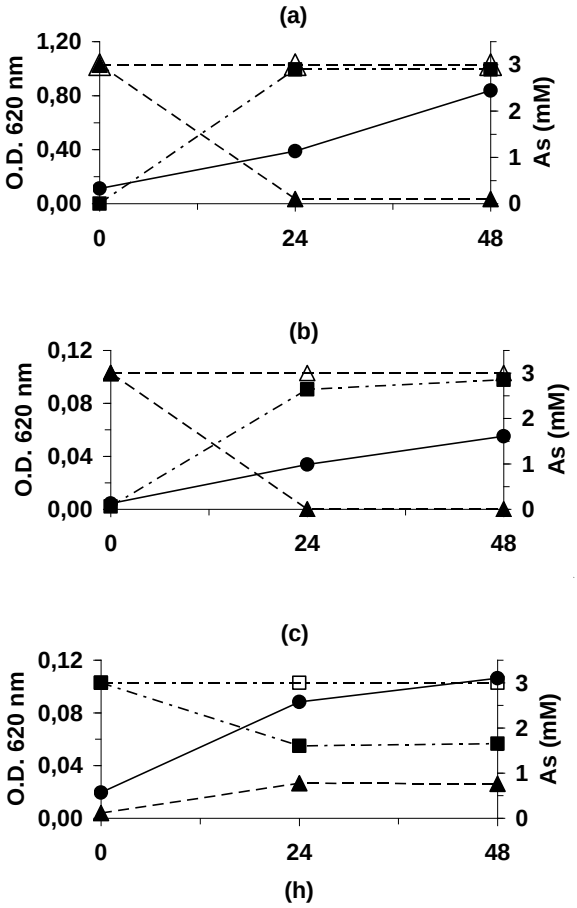
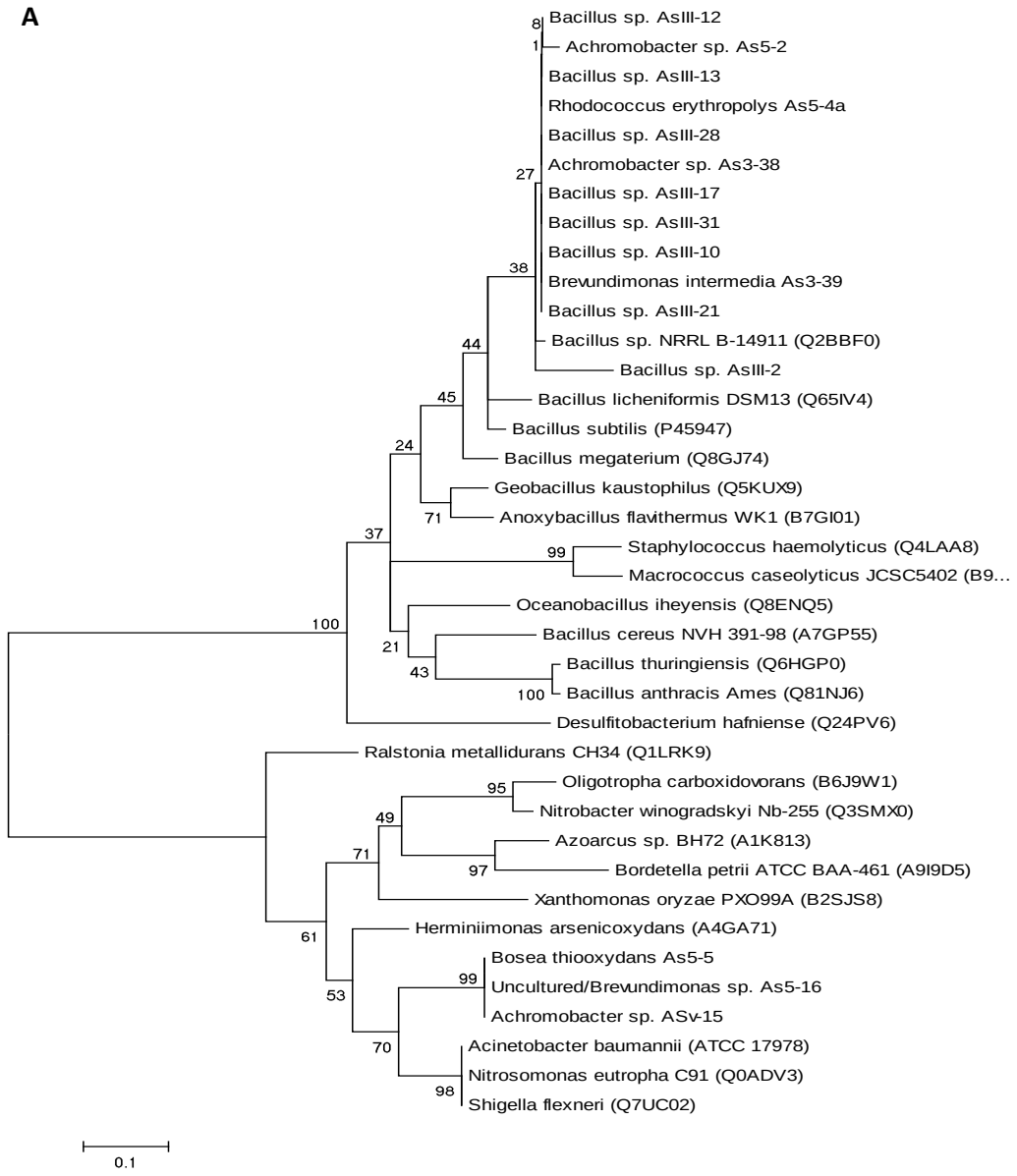
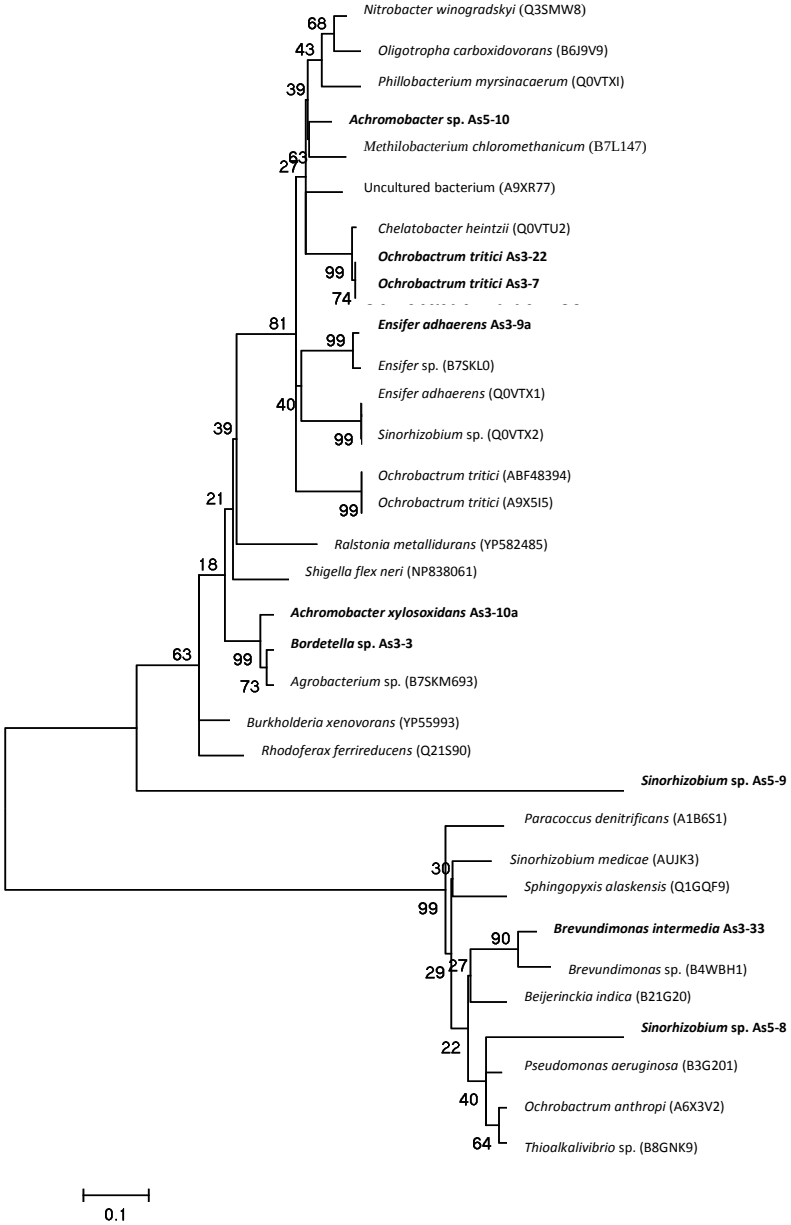


Fig. 4. Evolutionary relationships of arsenate reductase (ArsC) (A) and of arsenite efflux pump (ACR3) (B) deduced amino acid sequences retrieved from the isolates. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analysed. The evolutionary distances were computed using the Poisson correction method and are in units of the number of amino acid substitutions per site. Phylogenetic analyses were conducted in MEGA4 [59].


A
Bacillus sp. AsIII-12
Achromobacter sp. As5-2
Bacillus sp. AsIII-13
Rhodococcus erythropolys As5-4a
Bacillus sp. AsIII-28
Achromobacter sp. As3-38
Bacillus sp. AsIII-17
Bacillus sp. AsIII-31
Bacillus sp. AsIII-10
Brevundimonas intermedia As3-39
Bacillus sp. AsIII-21
Bacillus sp. NRRL B-14911 (Q2BBF0)
Bacillus sp. AsIII-2
Bacillus licheniformis DSM13 (Q65IV4)
Bacillus subtilis (P45947)
Bacillus megaterium (Q8GJ74)
Geobacillus kaustophilus (Q5KUX9)
Anoxybacillus flavithermus WK1 (B7GI01)
Staphylococcus haemolyticus (Q4LAA8)
Macrococcus caseolyticus JCSC5402 (B9...
Oceanobacillus iheyensis (Q8ENQ5)
Bacillus cereus NVH 391-98 (A7GP55)
Bacillus thuringiensis (Q6HGP0)
Bacillus anthracis Ames (Q81NJ6)
Desulfitobacterium hafniense (Q24PV6)
Ralstonia metallidurans CH34 (Q1LRK9)
Oligotropha carboxidovorans (B6J9W1)
Nitrobacter winogradskyi Nb-255 (Q3SMX0)
Azoarcus sp. BH72 (A1K813)
Bordetella petrii ATCC BAA-461 (A9I9D5)
Xanthomonas oryzae PXO99A (B2SJS8)
Herminiimonas arsenicoxydans (A4GA71)
Bosea thiooxydans As5-5
Uncultured/Brevundimonas sp. As5-16
Achromobacter sp. ASv-15
Acinetobacter baumannii (ATCC 17978)
Nitrosomonas eutropha C91 (Q0ADV3)
Shigella flexneri (Q7UC02)
0.1

B


Nitrobacter winogradskyi (Q3SMW8)
Oligotropha carboxidovorans (B6J9V9)
Phillobacterium myrsinacaerum (Q0VTXI)
Achromobacter sp. As5-10
Methilobacterium chloromethanicum (B7L147)
Uncultured bacterium (A9XR77)
Chelatobacter heintzii (Q0VTU2)
Ochrobactrum tritici As3-22
Ochrobactrum tritici As3-7
Ensifer adhaerens As3-9a
Ensifer sp. (B7SKL0)
Ensifer adhaerens (Q0VTX1)
Sinorhizobium sp. (Q0VTX2)
Ochrobactrum tritici (ABF48394)
Ochrobactrum tritici (A9X5I5)
Ralstonia metallidurans (YP582485)
Shigella flex neri (NP838061)
Achromobacter xylosoxidans As3-10a
Bordetella sp. As3-3
Agrobacterium sp. (B7SKM693)
Burkholderia xenovorans (YP55993)
Rhodoferax ferrireducens (Q21S90)
Sinorhizobium sp. As5-9
Paracoccus denitrificans (A1B6S1)
Sinorhizobium medicae (AUJK3)
Sphingopyxis alaskensis (Q1GQF9)
Brevundimonas intermedia As3-33
Brevundimonas sp. (B4WBH1)
Beijerinckia indica (B21G20)
Sinorhizobium sp. As5-8
Pseudomonas aeruginosa (B3G201)
Ochrobactrum anthropi (A6X3V2)
Thioalkalivibrio sp. (B8GNK9)
0.1

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IV

Role of PGP arsenic-resistant bacteria in As mobilization and translocation in *Helianthus annuus* L.

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Role of PGP arsenic-resistant bacteria in As mobilization and translocation in *Helianthus annuus* L.

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Abstract

This study investigated the effect of arsenic-resistant *Alcaligenes* sp. strain DhalL with potential plant growth promoting characteristics on growth and arsenic uptake by sunflower (*Helianthus annuus* L.). Pot experiments were prepared using an agricultural As-contaminated soil sown with seeds of sunflower uninoculated and inoculated with the strain. A Real Time PCR method, based on the quantification of *ACR3(2)* gene carried by DhalL, was set up in order to monitor presence and colonisation of the soil by the strain. The arsenic content was significantly higher in inoculated than in the uninoculated aboveground part of the plants (1.63 mg kg d.w.$^{-1}$ vs. 0.77), highlighting the effect of the strain on arsenic uptake by sunflower. *ACR3(2)* gene copy number was one hundred times higher in inoculated than in uninoculated pots, especially in the rhizospheric soil, indicating that colonisation occurred. The results suggest that the presence of arsenic resistant strain such as *Alcaligenes* sp. DhalL in the rhizosphere of sunflower could influence As mobilization and uptake by plant.

Key Words: Arsenic; *ACR3*; qPCR; PGPR; sunflower

Introduction

Arsenic (As) concentrations range from 1 to 40 mg As kg^{-1} in uncontaminated soil (Fitz and Wenzel, 2002). However, natural- and anthropogenic processes can elevate soil As levels. Arsenic mainly occurs in two inorganic forms, viz., arsenite (AsIII) and arsenate (AsV). Constituents of minerals, pH, redox potential, organic matter, interactions with Fe and Mn oxides, chemical speciation as well as biological activity affect the bioavailability and mobility of As in soil, enhancing As concentration in soil solution and causing toxic effect for plants (Adriano 2001; Islam *et al.* 2000, Bauer and Blodau 2006; Mukhopadhyay *et al.* 2002). To counteract the toxicity of As, microorganisms have evolved several mechanisms, that can be divided into different categories, consisting of detoxification reactions through the *ars* operon genes (Silver and Phung 1996), and of energy-gaining reactions through dissimilatory AsV reduction by a periplasmatic AsV-reductase and of AsIII oxidation by a AsIII-oxidase (Oremland and Stolz 2005). In plants arsenate, acting as an analogue of phosphate, is transported across the plasma membrane via phosphate co-transport systems while arsenite enters aspecifically through aquaglyceroporins. Some plants respond to biological- and environmental-stresses by synthesising ‘‘stress’’ ethylene from the precursor 1-amino-cyclopropane-1-carboxylic acid (ACC) (Morgan and Drew 1997). Plant growth-promoting rhizobacteria (PGPR) that produce the enzyme ACC deaminase cleave ACC and lower the level of ethylene, facilitating the formation of longer roots in plants growing in the presence of heavy metals (Grichko *et al.* 2000). Synergistic use of plants and metal-resistant PGPR is a promising approach for remediation of metal contaminated soils. Plants sequester the metals in their shoots, which can then be harvested. Different crop plants, among which sunflower (*Helianthus annuus* L.), can be used for phyto-extraction purpose because they can uptake

relatively high concentrations of metals and can have high yield biomass (Marchiol *et al.* 2007).

The objective of this study was to evaluate the effect of *Alcaligenes* sp. strain DhalL, an As-resistant strain with PGP characteristics, on the growth of *H. annuus* and on As translocation in the plant.

Methods

Experimental design

Pot experiments were set up using 250 g of soil sown with sunflower seeds (3 seeds in each pot). Five pots were sown with seeds treated according to Dell'Amico *et al.* (2008) with cell suspension (10^8 CFU ml^{-1}) of *Alcaligenes* sp. DhalL previously isolated from an As-contaminated soil (Bachate *et al.* 2009). Five additional pots represented uninoculated controls. Pots were placed in growth chamber with a 16/18 h photoperiod, 25°/18° C temperature, UR 80/60%. The soil under study was physico-chemically characterized according to the MIPAF Official Methods (2000). As fractions were determined by sequential extraction method (Wenzel *et al.* 2001).

Microbiological analysis

The number of total heterotrophic (THB) and As-resistant bacteria (ARB) in soil was determined by conventional plating techniques on 1/10 strength Tryptic Soy Agar (TSA/10) in the absence or in the presence of AsV (15 mmol l^{-1}) or AsIII (3 mmol l^{-1}), respectively. At 0, 27 and 50 days of incubation, rhizosphere soil samples were collected and divided into two fractions: the "bulk", obtained by manually shaking the roots, and the "rhizospheric" soil, obtained by washing the

roots with sterile distilled water. For each fraction the number of THB and ARB was determined. Strains were isolated from As plates and their As resistance level was determined by growth in Tris Mineral Medium supplemented with gluconate (0.6%, w/v) containing increasing amounts of AsV or AsIII. Strains were identified by 16S rRNA gene sequence analysis.

Molecular analysis and quantitative Real Time PCR

Genes *ArsC*, *ArsB*, *ACR3(1)* and *ACR3(2)* were amplified in the strains as reported in Bachate *et al.* (2009). At the different incubation times, DNA was extracted from bulk and rhizophere soil fractions (0.5g) by using UltraClean Soil DNA Isolation Kit (MOBIO, USA). Soil DNA was used in Real-Time quantitative PCR (q-PCR) experiments to quantify *ACR3(2)* gene, in order to monitor the presence of *Alcaligenes* sp. DhalL. Primer pair acr475F/611R (targeting a 137 bp fragment internal to *ACR3(2)* gene sequence of *Alcaligenes* sp. DhalL) and P1369F/1492R (targeting a 123 bp region of 16S rRNA gene) were designed. The reactions were set up with 1X SsoFast EvaGreen Supermix (BIORAD, USA). For generation of calibration curves of *ACR3(2)* and 16S rRNA genes, serial dilutions of total DNA extracted from *Alcaligenes* sp. DhalL were prepared. Relative quantification of *ACR3(2)* gene, normalized to 16S rRNA as reference gene (Livack Method, $\Delta\Delta$Ct) was performed in all the soil fractions.

Analytical methods

At 27 and 50 day of incubation, sunflower shoots dry biomass (105 °C) was measured and the As content was determined by ICP-MS (Inductively Coupled Plasma-Mass Spectrometry, Varian Inc.). Analytical data were compared by applying t-Student test using SPSS version 17.0 (Inc., Chicago).

Results

The soil used in this study was a loamy agricultural soil from Scarlino (Italy). The soil was moderately alkaline (pH 8.1), with a medium content of organic matter (11.5 g kg^{-1}) and a low content of available P (7.4 mg kg^{-1}). Total As content was 214 mg kg^{-1}: 181 mg kg^{-1} were associated with well crystallised Fe hydroxides, 25 mg kg^{-1} were specifically sorbed and 0.25 mg kg^{-1} were present as labile fraction. The soil contained THB in the order of 10^6 CFU g soil dw^{-1}, and AsV- and AsIII-resistant bacteria were 42% and 10% of the total heterotrophs, respectively. As evidence of the presence of As-resistant bacteria, several strains possessing *ArsB, ArsC, ACR3(1)* and *ACR3(2)* genes were isolated and characterized (Table 1). *Alcaligenes* sp. strain DhalL is an As-resistant strain (300 mM AsV and 70 mM AsIII) able to convert completely 2 mM AsV into AsIII within 72 hours (Figure 1). *Alcaligenes* sp. DhalL exerted an effect on As uptake by plant, as demonstrated by the higher As content in the inoculated than the uninoculated plants (Table 2). Although the strain possessed an ACC-deaminase activity (0.33 μmol $hr^{-1}\mu g^{-1}$), no significant difference on plant biomass was recorded, indicating that the strain did not promote sunflower growth (Table 2).

During the experiment, no significant difference in THB and ARB of the bulk fraction was recorded between inoculated and uninoculated pots (Table 2). On the contrary, in the rhizospheric fraction of inoculated pots THB and ARB were higher, suggesting that the strain colonized sunflower roots.

Although microorganisms with *ACR3(2)* gene were already present in the soil, q-PCR of *ACR3(2)* gene of *Alcaligenes* sp. DhalL was able to detect differences in inoculated and uninoculated soil DNA of bulk and rhizospheric fractions. Calibration curves (Figure 2) showed good correlation between DNA concentration (ranging from 0.026 to 6.5 ng μl^{-1}) of DhalL and fluorescence signal at each amplification cycle of *ACR3(2)* and of 16S rRNA genes. The melting curves for the

genes showed only one peak, indicating that no primer-dimers formed during the amplification steps. Data elaborated with the relative quantification Livak method (ΔΔCt), showed that at 27 days of incubation *ACR3(2)* gene was more abundant in the inoculated soil than in the uninoculated soil and it was higher in the rhizospheric than in the bulk fraction (Table 3). At 50 days, q-PCR data showed that strain DhalL multiplied, particularly in the rhizospheric fraction, indicating a preferential colonization of sunflower rhizosphere.

Conclusion

The results evidence that the potential PGPR *Alcaligenes* sp. DhalL colonized the sunflower rhizosphere and promoted the As uptake by plants. These data confirm that in soil As mobilization and uptake by plants would be influenced by the presence of As-resistant bacteria possessing an *Ars* operon (Meagher and Heaton 2005). As also reported by other authors (Marchiol *et al.* 2007), sunflower could be a candidate for phyto-extraction purposes and in a synergistic use with metal-resistant PGPR could represent a promising approach for remediation of metal contaminated soils.

Table 1. Characterization of bacterial strains isolated from the soil: arsenic resistance and presence of *ars* genes.

Isolates	As resistance[§] mmol l^{-1}		Gene fragments			
	AsIII	AsV	*ArsC*	*ArsB*	*ACR3(1)*	*ACR3(2)*
As3-1b *Ancylobacter dichloromethanicum* 98% EU589386	3	100	+	+	-	-
As3-1 *Brevundimonas intermedia* 99% FJ609705	25	100	-	-	+	-
As3-2b *Achromobacter xylosoxidans* 99% AF439314	3	100	-	+	-	-
As3-3 *Bordetella* sp. 99% FJ598334	10	100	-	+	-	+
As5-16 *Brevundimonas bacteroides* 97% AJ227782	5	200	+	-	+	-
As3-5a *Ensifer adhaerens* 100% FJ609715	3	10	-	-	-	+
As3-5 *Microbacterium* sp. 98% FJ595885	25	100	-	-	-	-
As3-8 *Ochrobactrum tritici* 100% EU870448	25	100	-	-	-	+
As3-9a *Ensifer adhaerens*	3	100	-	-	-	+
As5-11a *Microbacterium* sp. 99% FJ595885	5	200	-	-	-	-
As5-12 *Georgenia ferrireducens* 99% EU095256	2	200	-	-	-	-
As3-10a *Achromobacter xylosoxidans*	3	100	-	+	-	+
As3-10 *Bacillus* sp. 99% EU124558	50	100	+	+	-	-
As3-11 *Pseudomonas veronii* 99% FM162562	25	100	-	-	-	-
As5-3 *Sinorhizobium* sp. 99% AY505132	10	200	-	-	-	+
As5-4a *Rhodococcus erythropolis* 99% U81990	2	200	+	-	-	-
As5-4b *Bosea thiooxidans* 99% AJ250798	10	200	-	-	-	-
As3-15b *Ochrobactrum tritici*	25	50	-	-	-	+
As3-21 *Bacillus* sp. 99% EU124558	25	100	+	+	-	-
As3-22 *Ochrobactrum tritici* 100% EU301689	25	50	-	-	-	+
As3-25 *Ochrobactrum tritici* 100% EU301689	25	100	-	-	-	+
As5-8 *Sinorhizobium* sp.	10	200	-	-	+	+
As5-9 *Sinorhizobium* sp.	5	200	-	-	-	+
As5-10 *Achromobacter* sp. 99% EU073119	5	200	-	+	-	+
As3-30 *Bacillus* sp. 100% EU612335	50	100	+	+	-	-
As3-33 *Brevundimonas intermedia* 99% FJ609705	25	50	-	-	+	-
As3-35 *Microbacterium* sp. 99% FJ595885	25	50	-	-	-	-
As3-37 *Brevundimonas intermedia* 99% FJ609705	25	100	-	-	+	-
As3-38 *Achromobacter* sp. 100% EU073119	25	10	+	+	-	-

[§], the resistance to As(V) and As(III) was evaluated by an increase in OD_{620} nm (from twofold to fivefold) of the inoculum

Table 2. Total As content in shoots (mg kg^{-1} d.w.), aboveground plant biomass (g d.w.), counts of THB[A] and ARB[B] ($CFUg^{-1}$ d.w. soil).

Plants	Total As content		Plant biomass		THB				ARB			
					27 days		50 days		27 days		50 days	
	27 days	50 days	27 days	50 days	Bulk soil	Rhizospheric soil	Bulk soil	Rhizospheric soil	Bulk soil	Rhizospheric soil	Bulk soil	Rhizospheric soil
Inoculated	1.18*	1.96*	0.48	2.25	$5.60\pm0.94 \times 10^6$	$6.29\pm0.95 \times 10^5$	$2.90\pm0.47 \times 10^6$	$1.11\pm0.07 \times 10^7$	-	$6.32\pm2.34 \times 10^5$	-	$1.56\pm0.17 \times 10^6$
Uninoculated	0.77	1.76	0.46	1.84	$5.25\pm0.77 \times 10^6$	$4.50\pm0.45 \times 10^6$	$3.13\pm0.77 \times 10^6$	$1.13\pm0.18 \times 10^7$	-	$5.08\pm0.93 \times 10^5$	-	$1.25\pm0.23 \times 10^6$

*, statistically different from the uninoculated (t Student, p=0.1)
[A], total heterotrophic bacteria; [B], As-resistant bacteria

Table 3. Relative quantification of *ACR3(2)* in inoculated vs uninoculated pots, normalised to 16S rRNA calculated by Livak method, ΔΔCt (average ΔCt of inoculated pots – average ΔCt of uninoculated pots).

	T27		T50	
	Bulk soil	Rhizospheric soil	Bulk soil	Rhizospheric soil
ΔΔC(t)	70	690	148	400

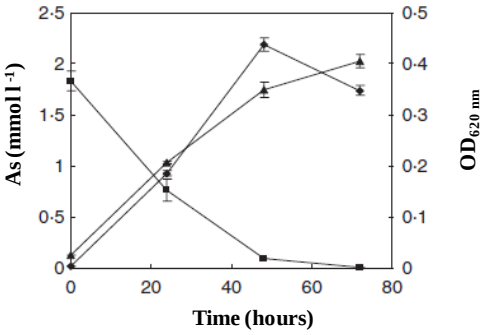

Figure 1. Bacterial growth (OD_{620nm}) (♦) and corresponding reduction of AsV (■) to AsIII (▲) by *Alcaligenes* sp. DhalL. The OD values reported are 1:10 dilution of original sample. Data are represented as means ± standard error, n = 4.

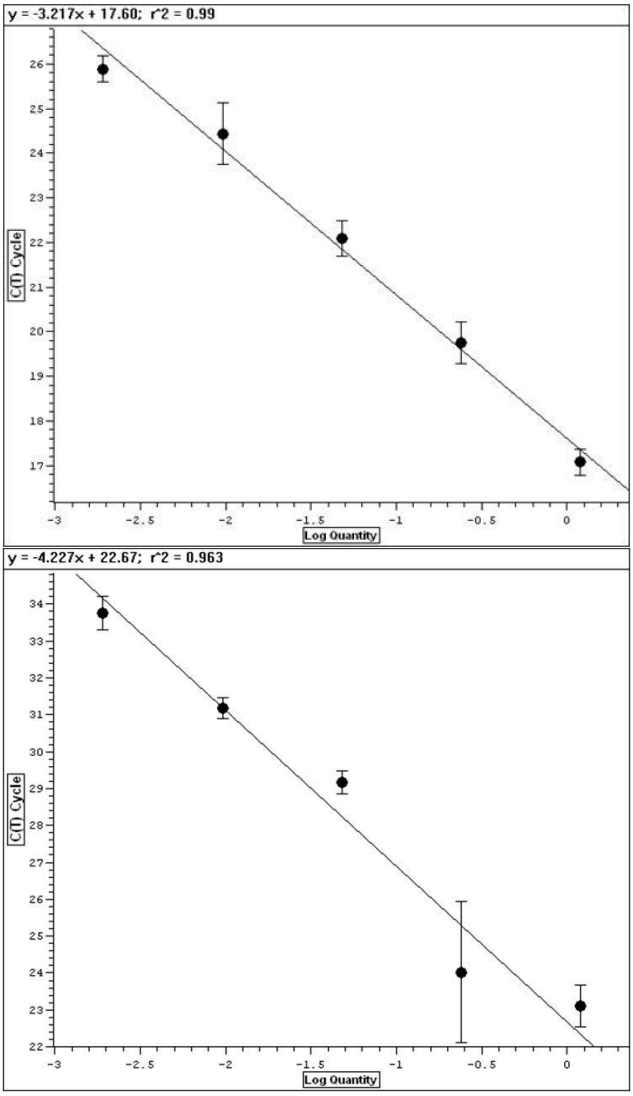

Figure 2. Calibration curves for 16S rRNA (A) and *ACR3(2)* (B) genes. C(t) values are plotted against Log of total DNA initial quantity (ng).

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V

Impact of glucose on microbial community of a soil containing pyrite cinders: Role of bacteria in arsenic mobilization under submerged condition

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Impact of glucose on microbial community of a soil containing pyrite cinders: role of bacteria in arsenic mobilization under submerged condition

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Abstract

Arsenic transformation and mobilization in a pyrite cinder-polluted soil were studied under submerged conditions both in the presence and absence of glucose. The presence of the carbon source enhanced bacterial activity and a reduction in the redox potential, resulting in release of higher amounts of arsenic iron and manganese in the aqueous phase. Since arsenic solubilization was not concomitant to that of iron, desorption rather than dissolution was found to be the main mechanism controlling its release from pyrite cinders. Arsenate was reduced to arsenite whose presence increased during the time course of the experiment. Denaturing gradient gel electrophoresis analysis of 16S rRNA genes of the total bacterial community revealed that the addition of glucose stimulated uncultivable populations of *Flavobacterium* and *Paenibacillus*. The isolation technique enabled the characterisation of nineteen arsenic resistant bacteria, mostly related to the facultative aerobic genera *Bacillus, Paenibacillus, Staphylococcus* and to *Rhodococcus* and *Micromonospora*. Most of them contained putative arsenate

reductase and/or arsenite efflux pump as indicated by the presence of *ArsC* and/or *ArsB* genes. Four strains showed the ability to reduce arsenate by an intracellular detoxification mechanism, and one strain was able to oxidize arsenite, indicating that bacteria with the ability to oxidize or reduce arsenic are ubiquitous in soils. The findings confirm that bacterial activity was responsible for the arsenic reduction causing the solubilisation of the metalloid from pyrite cinders to aqueous phases. Reducing conditions, such as those present in flooded soils in the presence of readily utilizable carbon sources could induce arsenic mobilization.

Keywords: Pyrite cinders; As-resistant bacteria; *Ars* genes; Bacterial redox activity; As solubilization; As(V); As(III)

Introduction

Arsenic (As) is present in high concentrations in soils due to natural- and anthropogenic processes and creates serious environmental concerns throughout the world. Arsenite As(III) and arsenate As (V) are the two inorganic forms mainly present in soils. The former is generally found under reducing environments while the latter predominates under well-oxidized conditions (Ackermann et al., 2008). However, due to slow redox transformations, both As(V) and As(III) are found in either environment. As(III) is less retained by soil colloids (Sadiq, 1997; Bissen and Frimmel, 2003a), while As(V) is preferentially adsorbed on positively charged surfaces like the (hydro)oxides of Fe Mn, and Al (Adriano, 2001; Fitz and Wenzel, 2002), the adsorption affinity being greater at low pH (Yang et al., 2002).

The bioavailability and mobility of As are governed by many physico-chemical and biological factors. Among factors affecting the equilibrium of precipitation and sorption reactions in nature, those playing determining roles are: parent mineral form and constituents of minerals, pH, redox potential, dissolved organic carbon

(DOC), competing ions, interactions with Fe and Mn oxides, and chemical speciation (Adriano, 2001; Al-Abed et al., 2007; Martin et al., 2007; Islam et al.,2000, Bauer and Blodau,2006).

However, microorganisms too play a significant role in speciation and geochemical behaviour of As (Macur et al., 2001; Routh et al., 2007; Bachate et al., 2009). Microbial reduction of As(V) can occur through a reductive intracellular mechanism of detoxification (Oremland and Stolz, 2003; Macur et al., 2004) or a dissimilatory reduction (respiration) (Oremland and Stolz, 2005). At first it acts on the *ars* operon which converts As(V) to As(III) that is pumped out of the cell, permitting the cells to survive in a highly As-contaminated environment without gaining energy. On the other hand, dissimilatory As(V) reduction usually occurs under anoxic conditions and microorganisms gain energy for growth by coupling As(V) reduction to oxidation of organic matter. Microbial oxidation of As(III) is carried out by heterotrophic (Gihring et al., 2001; Ehrlich, 2002) and chemoautotrophic bacteria (Santini et al., 2000; Oremland et al., 2002).

While heterotrophic As(III) oxidation is generally considered a detoxification reaction, chemoautotrophic oxidation provides energy and reducing power for CO_2 fixation and cell growth under aerobic (Santini et al., 2000) and nitrate-reducing (Oremland et al., 2002; Macur et al., 2004) conditions.

Whereas studies on the transport and outcome of As in terms of abiotic geochemistry are numerous (Chatain et al., 2005; Al-Abed et al., 2007; Signes-Pastor et al., 2007; Ackermann et al., 2008; Ascar et al., 2008; Noubactep et al., 2008; Phuong et al., 2008), those examining the effect of microbial processes on As behaviour in soil containing mineral processing wastes, for example of pyrite cinders, are few (Macur et al., 2001; Macur et al., 2004).

The research presented in this paper focuses on a soil polluted by pyrite cinders, which are a by-product of sulphuric acid manufacturing operations using pyrite

ores. Because these wastes were buried under a layer of loamy sand soil, the potential release of As from the cinders poses an environmental hazard.

The purpose of this work was to study the temporal dynamics of the bacterial community and the effect of its activity on soil redox potential and on As and Fe solubilization in a submerged experimental system spiked with glucose.

The specific objectives of this study were to: (i) investigate the influence of the added carbon source on bacterial As speciation and solubilization; (ii) identify microbial populations associated with the As transformations occurring in soil microcosms using cultivation-independent 16S rDNA sequence analysis; (iii) isolate As(V)-reducing- and As(III)-oxidizing aerobic heterotrophic bacteria from soil microcosms.

Materials and methods

Site description and soil sampling

The polluted area lies in the premises of a chemical factory that produced primary base- and fine-chemicals, located in the North-East of Italy (Torviscosa, Udine) and together with the surroundings is included in the national priority list of polluted sites (Decreto Ministeriale 468/2001). The soil of the site is contaminated by pyrite cinders, a by-product of sulphuric acid manufacturing operations (800°C roasting temperature). The cinders were deposited into the soil for about 40 years until the late 1970s. In the site, a horizon of pyrite cinders of about 1 m depth was covered by a layer of 0.2 m of carry-over, gravelly soil. In 2005, an experimental field was prepared in this area for phytoextraction experiments (Marchiol et al., 2007) by removing the covering soil of the field and mixing it with cinders in the same proportion (v/v). After three years had passed, a soil sample from the edges of the field as well as samples of the starting materials (pyrite cinders and covering soil) were collected, stored in sterile polyethylene bags, and transported to the

laboratory. First, the soil was sieved with 2 mm mesh size after which one part was air-dried for chemical analysis and another part was stored at 4°C for microbiological analysis and for set up of the microcosm.

Set up of microcosms

To examine the effect of the bacterial activity on As and Fe mobilization from the soil polluted with pyrite cinders, 50 g of soil were mixed with 50 g of water in 100-ml tubes; some of these tubes were then amended with glucose (0.2%, w/w) as carbon source and the rest were left unamended. 7.83 mg of Ca $(NO_3)_2$ was added to adjust the C/N ratio of glucose-amended microcosms to 30. The same amount of Ca $(NO_3)_2$ was also added to the unamended microcosms. Control soils were prepared by adding formaldehyde (0.04%, w/v) in the sample tubes to inhibit the microbial activity (Tuominen et al., 1994). The tubes were closed with cotton plugs and incubated statically at 30°C. Three sacrificial replicates of control, 0, and 0.2% glucose thesis were prepared for each sampling time. Time courses of pH, redox potential, total As, As(V), As(III), Fe contents in the aqueous phase were determined at successive incubation days. Soluble Mn content was checked on glucose-amended and unamended microcosms to evaluate the potential role of Mn oxides on As and Fe transformation and sorption. The counts of aerobic and anaerobic heterotrophic bacteria as well as total DNA extraction were also carried out.

Chemical and microbiological determinations

Polluted soil was chemically and physically characterized in accordance with MIPAF Official Methods (2000). pH_W was measured in a soil suspension with a solid:water ratio of 1:2.5 (w/v). Texture was defined with sand (50–2000 μm), silt

(2–50 μm), and clay (<2 μm) fractions. Exchangeable cations were extracted with a barium chloride-triethanolamine solution at pH 8.1. To determine the total content of heavy metals and As, the samples were HNO_3/HCl-digested in a microwave oven (CEM, MARS5); the slowly labile fraction of heavy metals was determined by diethylene triaminepentaacetate (DTPA) extraction. Water soluble fraction of As was determined with a solid: water ratio of 1:1 (w/v). To perform As fractionation of the soil, a five-step sequential extraction procedure described by Wenzel et al. (2001) was applied. This technique graded the occurrences of As as non-specifically sorbed, specifically-sorbed, associated with amorphous and poorly-crystalline (hydro)oxides of Fe and Al, associated with well-crystallized hydrous oxides of Fe and Al, and residual phases.

Redox potential and pH in microcosms were measured in the upper aqueous phase of the tubes, retaining the electrodes until the measurements were stabilized. The tubes were successively shaken for 10 min and then centrifuged at 6000 rev min^{-1} for 15 min. The supernatant was collected, filtered (0.45 μm), and stored at -20°C until the determination was completed. As, Fe and Mn contents in the solution were determined in 10 ml of supernatant after acidification with HCl to pH 2.0. As(III) and As(V) contents were determined in 10 ml of supernatant passed through a WATERS Sep-Pak® Plus Acell Plus QMA cartridge (Waters, MA). As(V) was retained in the cartridge while allowing As(III) to pass through and collected. The cartridge was then washed with 0.16M HNO_3 to extract As(V) from it (Kim et al., 2007).

Exchangeable cations, heavy metals, Fe, Mn and As contents were determined by ICP-MS (Varian Inc.). Standards of As for concentrations ranging from 0-1 mg l^{-1} were prepared from sodium arsenite ($NaAsO_2$) (Sigma-Aldrich) solution.

The number of heterotrophic bacteria in the polluted soil was determined using conventional plating techniques. Triplicate samples of soil (3 g) were suspended in 27 ml of sodium pyrophosphate solution (0.2%, w/v), shaken in a rotary shaker at

180 rev min^{-1} for 1 h and left to rest for 10 min. Aliquots (1 ml) of the soil suspensions were serially diluted 10-fold in a physiological solution (0.9% NaCl) after which 1 ml was plated on 1:10 diluted Tryptic Soy Agar (TSA/10) in double sets of plates supplemented with sterile cycloheximide (1% w/v) to inhibit fungal growth. The incubation period was 10 days at 30°C. Similarly, the number of As-resistant bacteria was determined on TSA/10 medium supplemented with 10 mmol l^{-1} of sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$, Sigma-Aldrich) or 3 mmol l^{-1} $NaAsO_2$. The As-resistant heterotrophic bacteria detected were expressed as percentage of growth on TSA/10 without As addition.

In the microcosm experiments, the number of aerobic- and anaerobic heterotrophs was determined in each of the samples of unamended and glucose-amended microcosms and in controls. At each sampling time, soil suspensions of 1 ml aliquots each were serially diluted and plated on TSA/10 as described above. Plates of aerobic bacteria were incubated at 30°C for 10 days, while the plates for anaerobic bacteria were incubated in anaerobic jars in the presence of Anaerocult® A (Merk) at 30°C for 15 days.

Isolation and characterization of aerobic As-resistant bacteria

To isolate arsenic-resistant bacteria, appropriate serial dilutions of soil suspensions from glucose-amended microcosms at 7 and 15 days of incubation were plated both on TSA/10 containing 10 mmol l^{-1} of As(V) and on 3 mmol l^{-1} of As(III). After 10 days of incubation at 30°C in aerobic conditions, the colonies were randomly isolated from plates containing As(V) or As(III). Single colonies were streaked to purity on the same medium. Strains were maintained in glycerol stocks at -70°C.

These isolates were successively characterized for their resistance to As(V) and As(III) and their capability to transform As. Prior to use, the strains were grown to mid-exponential phase in liquid Tris Mineral Medium (TMM) of low phosphate

content (Mergeay et al., 1985), supplemented with 0.6% (w/v) gluconate (TMMG) at 30°C and kept shaken at 180 rev min^{-1}. Resistance to As species was determined by growing the isolates in 20 ml TMMG supplemented with increasing concentrations of either As(V) (from 0 to 100 mmol l^{-1}) or As(III) (from 0 to 10 mmol l^{-1}) and checking for growth after 72 hour of incubation. To test the ability of the isolates to reduce As(V) or to oxidize As(III), each strain was inoculated into three vials each containing 20 ml of TMMG supplemented with 1 mmol l^{-1} As(V) or As(III). Three vials with no As content were inoculated to compare the growth of the microorganisms in the absence of As(V) or As(III). Three vials were also prepared without inoculum in order to check for abiotic transformation of arsenic. At each sampling time, 2 ml of cell suspensions were removed to follow cell growth with OD_{620nm} and to determine As(V) and As(III) concentrations by spectrophotometric analysis according to the method of Dhar et al. (2004). This method was slightly modified by lowering the concentration of ascorbic acid from 613.0 mmol l^{-1} to 306.5 mmol l^{-1} and of antimony potassium tartrate from 8 mmol l^{-1} to 2 mmol l^{-1}. Standards were prepared for concentrations ranging from 0–1000 µmol l^{-1} for both As(V) and As(III) from $Na_2HAsO_4 \cdot 7H_2O$ and $NaAsO_2$ solutions, respectively.

Identification of the isolates

DNA was extracted from TMMG liquid cultures grown overnight with Proteinase K digestion (1 mg ml^{-1}). Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was performed on the extracted DNA using the eubacterial universal primers P27f and P1495r referred to *Escherichia coli* nucleotide gene sequence according to Weisburg et al. (1991). PCR reactions were performed in a final volume of 50 µl containing 10 ng of DNA, 1.5U of *Taq* polymerase, 0.2 µmol l^{-1} of each primer, 0.2 mmol l^{-1} of dNTPs, 1.75 mmol l^{-1} of $MgCl_2$, and 1X PCR buffer.

DNA amplification conditions were: initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 1 min, 55°C for 40 s, 72°C for 1 min 40 s and then a final extension step at 72°C for 10 min. Isolates were clustered into five operational taxonomic units (OTU) on the basis of amplified ribosomal DNA restriction analysis (ARDRA) performed with *Hha*I, *Hae*III, and *Alu*I enzymes according to manufacturers' instructions (Invitrogen). A selection of representative isolates of each OTU was chosen for 16S rRNA gene sequencing.

Arsenic resistance gene amplification

Primer P52f (5'-AGCCAAATGGCAGAAGC-3') and P323r (5'-GCTGGRTCRTCAAATCCCCA-3') were used for arsenate reductase *ArsC* amplification according to Bachate et al. (2009). Primer darsB1F (5'-TGTGGAACATCGTCTGGAAYGCNAC-3') and darsB1R (5'-CAGGCCGTACACCACCAGRTACATNCC-3') were used to amplify arsenite efflux pump *ArsB* (Achour et al., 2007). Degenerate nucleotide sites are indicated by standard ambiguity codes as follows: N= A, C, G, or T; R= A or G; V= A, C, or G and Y= C or T. PCR reactions were performed in a final volume of 25 µl containing: 10 ng total DNA, 0.2 mM of dNTPs, 1.75 mM of $MgCl_2$, 0.4 µM of each primer, 2U of *Taq* polymerase, and 1x PCR buffer. DNA amplification condition for the P52f/P323r pair was: initial denaturation at 94°C for 5 min, 40 cycles of 94°C for 45 sec, 55°C for 45 sec, 72°C for 30 sec and then a final extension step at 72°C for 7 min. DNA amplification conditions for primer sets darsB1F/darsB1R were as described by Achour et al. (2007). All reagents were obtained from Invitrogen. PCR reactions were performed on T-Gradient Biometra apparatus. The PCR products were checked on 2% agarose gel and visualized by ethidium bromide staining using the GelDoc image analyzer system (Biorad).

Denaturing gradient gel electrophoresis (DGGE) analysis

Dynamics of the bacterial communities related to the addition of glucose was studied at successive incubation times (0, 7, and 15 days) in triplicate samples with DGGE applied to the hyper variable region V3 of bacterial 16S rRNA gene. Total DNA was extracted from soil microcosms using a bead-beating method (MOBIO, USA) according to manufacturer's instructions. PCR amplification of V3 regions was carried out using a nested PCR approach: the 16S rDNA amplicons, obtained as described for isolates, were used as templates for a second amplification step of the V3 internal fragment by primer pair V3-GC clamped forward and V3 reverse (Muyzer et al. 1993). PCR reactions were performed in a final volume of 50 µl containing the following: 10 ng of DNA, 1.5 U of *Taq* polymerase, 0.3 µmol l^{-1} of each primer, 0.2 mmol l^{-1} of dNTPs, 1.75 mmol l^{-1} $MgCl_2$, and 1X PCR buffer. The samples were first denatured for 3 min at 94°C and then subjected to 19 cycles consisting of 1 min 20 s at 94°C, 1 min 30 s at 65°C, and 1 min 10 s at 72°C; the annealing temperature was reduced by 1°C every second cycle until touchdown at 55°C, at which temperature 7 additional cycles were carried out. The last step included an extension of 5 min at 72°C. V3-GC PCR amplicons were then loaded onto polyacrylamide gels (8%) in a DCODE Universal Mutation Detection System (Biorad) apparatus. The linear denaturing gradient of urea and formamide ranged from 40% (top) to 60% (bottom) where 100% denaturant gels contained 7 M urea and 40% formamide. Electrophoresis was performed on samples (10 µl) at a constant voltage of 70 V for 16 h in 1x TAE (4.84 g l^{-1} of Tris base, 1.14 ml l^{-1} of glacial acetic acid, 2 ml l^{-1} of 0.5 mol l^{-1} EDTA solution of pH 8.0) running buffer at 60°C. After completion of electrophoresis, gels were stained in SYBR Green 1 solution according to manufacturer's instructions (Molecular probes, USA) and documented with the GelDoc System (Biorad). DGGE bands of interest were excised with a sterile scalpel, suspended in sterile Milli-Q water, and used as

template for V3 amplification by primers V3 forward without GC clamp and V3 reverse.

Sequence analysis

16S rDNA and arsenic resistance genes and DGGE DNA bands were sequenced using the *Taq* Dye-Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, USA) with the respective primers. The forward- and the reverse samples were run on a 310A sequence analyser (Applied Biosystems, USA). Sequences were compared with the entire GenBank/EMBL nucleotide and amino acid databases using the BlastN and BlastX query programs (http.//www.ebi.ac.uk/blastall/index.html).
Sequences were deposited under the accession numbers from FN429085 to FN429102.

Statistical analysis

Analytical data from the microcosm experiments were compared applying ANOVA and the b-Tukey test using SPSS version 17.0 (Inc., Chicago).

Results

Chemical- and microbiological characteristics of the soil

Pyrite cinders and covering soil were characterized for some relevant agronomic parameters and for total As. Since in pyrite cinders As coexisted with iron and heavy metal, their contents were determined. Pyrite cinders had neutral pH (6.9), no organic matter, and a negligible amount of total carbonates; they were silty

loam, with a prevalence of fine silt that implied a high susceptibility to cementation and a low permeability. The covering soil which was moderately alkaline (8.2), was loamy sand with a high content of gravel, a medium content of organic matter (14.6 g kg^{-1}), and low amounts of carbonate, As, and heavy metals.

As shown in Table 1 the resulting polluted soil was sandy loam, subalkaline, with a low content of organic matter and available P and low Cation Exchange Capacity (CEC). The soil contained heterotrophic bacteria in the order of 10^6 CFU, and As(V)- and As(III)-resistant bacteria were 58% and 33% of the heterothrophs, respectively (Table 1).

Pyrite cinders were constituted mainly of hematite (Alp et al., 2009) and contained iron arsenate formed during the roasting process (Ciobanu, 1994). Soil pollution was related to the presence in the pyrite cinders of high amounts of As, Cd, Cu, and Zn. Heavy metal bioavailability, with the exception of Cu, was less than 5% of the total amount due to the thermal treatment of pyrite. The prolonged contact of cinders with soil raised bioavailability of Cu but not those of Cd and Zn (Table 2). Soluble As in the soil represented a negligible part of the total amount. The sequential extraction procedure (Table 3) showed that As was present mainly in the residual phase (nearly 50%), and associated with Fe hydroxides (26%) in the crystalline and amorphous forms (17%). The more labile fraction of As (non-specifically-sorbed As) accounted only for 1.5% of the total As present.

Effect of glucose on the bacterial community of microcosms

Figure 1(A and B) shows the dynamics of the bacterial counts carried out during the experiment. In glucose-amended microcosms, the number of anaerobic bacteria present was 1-2 orders of magnitude lower than that of aerobic bacteria, indicating that a fraction of the former, probably the anaerobic respiring bacteria, did not grow in TSA/10 medium for want of appropriate electron acceptors. A slight but

significant increase of anaerobic heterotrophs was observed at day 7 after which their number did not apparently change. Whereas, it was observed that at day 15 the utilization of glucose or its derivatives resulted in an increase in the number of aerobic heterotrophs. The effect of formaldehyde on the bacterial population of the controls was evident, resulting in a reduction of the number of either the aerobic or anaerobic heterotrophs (data not shown).

The DGGE profiles of total soil bacteria in unamended microcosms did not alter during the incubation period, indicating that the structure of the bacterial community was not affected by the submerged conditions. On the contrary, glucose addition brought about noticeable modifications, as evidenced by the increment of five bands ("a, b, c, d, e") and by the disappearance of one band "f"(Fig. 3) at day 7 and day 15. A similarity analysis of the nucleotide sequence of the bands towards the 16S rRNA gene sequence databases showed that the five predominant bands belonged to four different uncultured *Flavobacterium* sp. strains (100% identity of nucleotide sequence to Acc. Numbers AM230410, EU097093, EF378178, and EU297120, respectively) and to one uncultured microorganism of *Paenibacillus* sp. (100% identity to Acc. Number DQ839177). Band "f" was related to *Beggiatoa* sp. (84% to Acc. Number L40994) and its disappearance could be related to the conditions developed after glucose addition that probably affected this bacterial population.

Effect of glucose on pH, redox potential, As, and Fe solubilization in microcosms

A decrease of 0.6 units of pH of the aqueous phase of glucose-amended microcosms occurred in the first 15 days of incubation, after which pH went back to the initial value (Fig. 2A). However, in controls and in unamended microcosms pH rose slightly but significantly during the experiment. In glucose-amended microcosms, redox potential gradually declined from 300 mV to - 42 mV over the

passage of 30 days (Fig. 2B), unlike in the controls and unamended microcosms where it remained constant.

As, Fe and Mn mobilization from soil to the aqueous phase occurred only in glucose amended microcosms. As and Mn were more readily solubilized (day 7) than Fe (day 15). However, As and Fe at day 30 appeared to be partially re-immobilized (Table 4).

As speciation in glucose-amended microcosms

In glucose amended-microcosms the amount of As(III) going into solution increased with time until the 15 day, rising from 0.14 to 1.1 mg kg^{-1}. However, at day 30 , it was found to be 0.65 mg kg^{-1}, indicating that almost 40% of As(III) formed was re-adsorbed. A small but significant increase of As(V) was also measured (Fig. 4), whereas in unamended microcosms the amount of As(III) and As(V) remained constant throughout the experiment.

Identification of aerobic As-resistant isolates and As transformation patterns

Nineteen As-resistant bacteria were isolated from glucose-amended microcosms, at day 7 and day 15 of incubation. These isolates belonged to Firmicutes (16 strains) and to Actinobacteria (3 strains), while no Proteobacteria was isolated. The As resistance patterns varied from high to medium: *Paenibacillus* sp. strain 3.1 exhibited the lowest resistance to As(III) (3 mmol l^{-1}), while *Staphylococcus xylosus* strain 5.2 and *Bacillus* sp. strain 3.9 showed the lowest resistance to As(V) (25 mmol l^{-1}).

ArsC genes for arsenate reductase and/or *ArsB* for the arsenite efflux pump were successfully detected in 15 isolates (Table 5). A 275 bp fragment of *ArsC* genes was amplified in most of the *Bacillus* strains and in *Rhodococcus gordoniae* strain

5.6. Deduced aminoacid sequence analysis of *ArsC* amplicons of strains *Bacillus* sp. 3.2 and *Rhodococcus gordoniae* 5.6 revealed that they were highly homologous (88% and 92% identity, respectively) to the same arsenate reductase ArsC of *Bacillus cereus* strain AH820 (B7JU52). *ArsC* fragments retrieved in *Bacillus humi* 5.3 and *Bacillus licheniformis* 5.5 were highly homologous to, respectively, the ArsC of *Bacillus megaterium* (85% identity to Q8GJ74) and of *Bacillus* sp. NRRL B-14911 (84% identity to Q2BBF0). *ArsB* was detected in 5 isolates, while the *ACR3* gene was not recorded in any of the isolates. Deduced amino acid sequence analysis indicated that an arsenite efflux pump highly homologous to YdfA of *Bacillus* sp. NRRL B-14911 was present in the strains (83% of identity to Q2BBE9).

Eight isolates were tested for their capability to reduce As(V) or oxidize As(III). *Paenibacillus* sp. 3.1, *Staphylococcus xylosus* 5.2, *Bacillus* sp. 3.2 and 5.8 reduced completely 1 mmol l^{-1} of As(V) in 24 h under aerobic conditions (Fig. 5A, B, C, D). Complete reduction of As(V) occurred when the strains attained maximum cell density. On the contrary, *Rhodococcus* sp. 3.3, *Rhodococcus gordoniae* 5.6, *Bacillus* sp. 3.9 and 5.1 did not reduce arsenate. With the exception of *Bacillus* sp. 3.9, which oxidized 1 mmol l^{-1} of As(III) in 72 h, the other isolates did not oxidize As(III). In particular, strain 3.9 grew in the presence of As(III) at a significantly higher rate than in the absence of As(III) (Fig. 5E). Arsenic transformations did not occur in controls with no cells, indicating that the processes were due to microbial activities.

Discussion

The polluted soil under study showed low nutrient and organic matter content and low bioavailability of contaminants. The numbers of heterotrophs encountered were higher than those reported for heavily polluted soils (Roane and Pepper,

1999; Brim et al., 1999), probably due to the bacterial charge of the unpolluted covering soil. A substantial proportion of the culturable bacteria resisted the presence of As(V) and As(III). This indicated that these populations had been under constant metalloid stress for long, As having exerted a selective pressure upon them. The large number of As(III)-resistant bacteria in the soil can be ascribed to the predominance of arsenite in the water-soluble fraction (70% of total soluble As). It is known that As(III) can be present under aerobic conditions too (Macur et al., 2001; Macur et al., 2004).

In submerged microcosms, the microbial activity enhanced by glucose addition induced marked changes in redox potential. Free oxygen was initially consumed by respiring microorganisms that drove the microcosms towards anaerobic conditions. Facultative anaerobic and anaerobic bacteria, such as denitrifying flavobacteria, enhanced during incubation, reduced the alternative electron acceptors (NO_3^-, Mn^{4+}, Fe^{3+}), causing a further decrease of the soil redox potential.

The presence of glucose enhanced the leaching of As, Fe and Mn from the soil by stimulating bacterial activity, because it represented an electron donor and a carbon source. Moreover it is recognized that DOC can influence the adsorption processes of As in soils (Mohapatra et al., 2007). Anawar et al. (2006) found that glucose had an important role in As and Fe mobility in reducing condition and demonstrated the biological nature of the process by autoclaving and poisoning the glucose-amended sediments. In the present study, similarly glucose and microbial derivatives may induce a biological As and Fe leaching rather than directly interact with As adsorption processes.

The increased solubilization observed could be explained by the reduction of As(V) and Fe(III) (Lovely, 1993; Zobrist et al., 2000): As(V) would be reduced under aerobic conditions by aerobic bacteria via the Ars detoxification system (Macur et al, 2004; Bachate et al., 2009) and later, when anaerobic conditions became predominant, via dissimilatory reductions by anaerobic respiring bacteria.

Arsenate-respiring bacteria have been isolated from different environments and were believed responsible for release of As from ferric and aluminium (hydro)oxides (Zobrist et al., 2000;Oremland and Stoltz, 2003; Islam et al., 2004). The biological reactivity of arsenic observed in the submerged studied soil was mainly related to redox modifications rather than to the slight acidification of the aqueous phase at neutral pH.

Solubilization of Fe and As followed two different kinetics: while that of As was rapid and occurred under moderately reduced conditions (around +100 mV) (Reedy et al., 2000), that of Fe was delayed, requiring greater reduced conditions (0 mV). An uncoupled release of Fe and As was generally observed. While Islam et al. (2004) and Solaiman et al. (2009) observed that in natural and artificial sediments mobilization of Fe preceded that of As, other authors observed that in sediments mobilization of As occurred first and that of Fe followed (Radlof et al., 2007; Van Geen et al., 2004). These contradictory findings suggest that the relationship between As- and Fe release is complex. Since in our experimental conditions Fe solubilization was not concomitant to that of As, desorption rather than dissolution was the main mechanism controlling release of arsenic from pyrite cinders.

Given that the amount of solubilized As was lower than that non-specifically sorbed, mobilization of As could be attributed to the fraction adsorbed on Fe (hydro)oxides (hematite) generated during the industrial pyrite treatment.

Soluble As is strongly governed by the presence of As(III), which was the predominant species present in glucose-amended microcosms.

The evidence that about 50% of the As(III) formed was re-adsorbed by the iron oxides, is in accordance with data of Dixit and Hering (2003) which found that As(III) is sorbed to a similar or greater extent than As(V) on iron oxides. Finally, the increase of As(V) observed at day 30 was due to increase in solution pH that could have led to As(V) desorption.

In natural systems, manganese oxides can play a role on As(III) and Fe(II) oxidation (Oscarson et al., 1983; Manning et al., 2002; He and Hering, 2009). Mn oxides are more susceptible to reductive dissolution under slight to moderate soil reductive conditions than Fe oxides and consequently Mn dissolution precedes Fe dissolution in saturated soils (Patrick and Jugsujinda, 1992). In the studied glucose-amended microcosms, the dissolution of Mn oxides that derive mainly from covering soil occurred in the early incubation time (7 day) thus suggesting a negligible role of Mn oxides on As and Fe behaviour. However, in field condition where oxidizing environment may be restored, Mn oxides can interact with Fe and As affecting their precipitation/ sorption (Amirbahman et al., 2006).

DGGE analysis revealed conspicuous shifts of the bacterial populations related to the addition of a readily utilizable carbon source. Glucose addition apparently caused the disappearance of the *Beggiatoa* population, which was possibly sensitive to the new physico-chemical conditions, among which increased concentrations of As(III), and the enrichment of *Flavobacterium* and *Paenibacillus* populations, often retrieved in As-contaminated soils (Macur et al., 2004; Bachate et al., 2009). Particularly, *Flavobacterim* is a genus known to take advantage of glucose utilization and comprises aerobic-, anaerobic-, or denitrifying strains. Nucleotide sequences of intense DGGE bands were related to uncultivated environmental clones, being only the *Paenibacillus* population mirrored by the isolation of one As-resistant strain. Clearly, intense bands of DGGE patterns do not always reflect the most abundant, the most active, or the most As-resistant species of the culturable fraction, but highlight modifications of bacterial populations in relation to environmental changes. The culturable approach allowed characterising a greater number of As-resistant species, indicating that both the approaches are relevant in depicting bacterial communities in soils.

In the present work, As-resistant isolates were mostly related to facultative anaerobic genera. Firmicutes were the most abundant strains and most of them

carried an ars detoxification system. The arsenic resistance pattern observed was a strain-dependent trait, representing a possible case of plasmid-borne transmission within bacterial populations. Interestingly, the *ArsC* gene of *Bacillus* sp. 3.2 and *Rhodococcus gordoniae* 5.6 were highly homologous, possibly evidencing a case of transmission.

Four isolates, namely *Staphylococcus xylosus* 5.2, *Paenibacillus* sp. 3.1, *Bacillus* sp. 3.2, and 5.8, reduced completely 1 mmol l^{-1} As(V) to As(III) under aerobic conditions. Although the isolates were never tested for dissimilatory As(V) reduction, the concomitance of As(III) formation and microbial growth suggested that the mechanism was related to As detoxification. The As(V)-reduction ability of strain 3.1 was not related to the presence of such a gene system, indicating that the primers used did not take into account sequence variations present in the strain or that an alternative As-transforming system could be present. None of these strains oxidized As(III), possibly due to the lack of genes required for As(III) oxidation or for the repression of the As(III) oxidation pathway under the conditions studied here. As(III) was instead oxidized by *Bacillus* sp. 3.9. The significant higher growth of this strain in presence of As(III) than in its absence suggests that the mechanism of As(III) oxidation in *Bacillus* sp. 3.9 could be related to energy generation rather than to As detoxification. This strain, as well as *Bacillus* sp. 5.1 and *Rhodococcus gordoniae* 5.6, did not exhibit As(V) reduction, despite carrying an *ArsC* gene. The apparent lack of As(V) reduction may be due to different reasons including point mutation in *ArsC* or separation of *ArsC* from its As derepressible promoter. The isolation of both As(V)-reducing and As(III)-oxidizing strains from the same soil showed that the ability to oxidize As(III) or to reduce As(V) is variable even among strains that proliferate under the same environmental conditions (Macur et al., 2004). The fact that both oxidizers and reducers were obtained from the same microcosms suggests that both processes might have been occurring simultaneously.

Results from the current study as well as others recently reported (Garcia-Dominiquez et al., 2008; Macur et al., 2004; Romagnoli et al, 2009, submitted) support the hypothesis that the oxidation and the reduction of As occur in phylogenetically diverse soil bacteria via mechanisms that are not directly associated with respiration or chemiolithotrophic metabolism.

Conclusions

The results obtained are of environmental relevance because of the possibility that the bacterial activity of the soil could initiate the reduction of As(V) contained in pyrite cinders to As(III), causing the mobilization and transport of arsenic to the surrounding surface water and groundwater. Since As mobilization does not require the dissolution of iron hydroxides, it is possible that such a process could occur even in polluted soil containing different As-bearing phases such as Al-oxide and phyllosilicate (Violante et al.,2006).
Flooding of soils containing a readily utilizable carbon source might induce soil redox potentials that allow mobilization of As, which can be up taken by plants and eventually translocated into the food chain.

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Table 1. Physical, chemical, and microbiological characteristics of the soil (mean ± standard deviation, n=3)

pH_W		7.75
Organic carbon (g kg^{-1})		9.14
C.E.C. ($cmol^{(+)}$ kg^{-1})		2.34
Olsen P (mg kg^{-1})		7.78
Exchangeable K (mg kg^{-1})		132
Gravel (%)		33
Sand (%)	2 - 0.05 mm	72.4
Silt (%)	0.05-0.002 mm	25.6
Clay (%)	< 0.002 mm	2.0
Aerobic heterotrophs (CFU g^{-1} dry soil)		4.78 ±1.04 x 10^6
Anaerobic heterotrophs (CFU g^{-1} dry soil)		1.42 ± 0.17 x 10^5
Aerobic As(V)-resistant heterotrophs (CFU g^{-1} dry soil)		1.95 ± 0.25 x 10^6
Aerobic As(III)-resistant heterotrophs (CFU g -1 dry soil)		1.15 ± 0.12 x 10^6

Table 2. Total and bioavailable arsenic and heavy metals in pyrite cinders and in soil (mg kg^{-1})

		Pyrite cinders	Polluted soil
Fe	Total	600*	318*
Mn	Total	25	82
As	Total	750	446
	Water sol.	n.d.	0.17
Cd	Total	9.61	5.53
	DTPA sol.	0.252	0.11
Cr	Total	13.9	18.6
	DTPA sol.	< 0.5	n.d.
Cu	Total	1971	1527
	DTPA sol.	121	228
Ni	Total	20.2	22.9
	DTPA sol.	< 0.2	0.22
Pb	Total	410	255
	DTPA sol.	71.8	13.8
Zn	Total	1876	980
	DTPA sol.	55.80	23.64

n.d. – not detected
* Fe concentration is in g kg^{-1}

Table 3. Partitioning of As (mg kg^{-1}) in soil fractions (mean ± standard deviation, n=3)

Non-specifically sorbed	Specifically sorbed	Amorphous and poorly-crystalline hydrous oxides of Fe and Al	Well-crystallized hydrous oxides of Fe and Al	Residual phase
3.51±0.25	29.6±1.14	75.7±1.27	114±3.89	224±2.94

Table 4. As, Fe and Mn (mg kg^{-1} dry soil) in the aqueous phase of controls, unamended and glucose-amended microcosms during a 30-day incubation period (n=3).

	Arsenic (mg kg^{-1})				Iron (mg kg^{-1})				Manganese (mg kg^{-1})		
	Day 0	Day 7	Day 15	Day 30	Day 0	Day 7	Day 15	Day 30	Day 0	Day 7	Day 15
Control	0.171	0.149 A	0.137 A	0.153 A	1.14 ab	1.06 a	1.10 A ab	1.34 A b	n.d.	n.d.	n.d.
Unamended	0.165	0.173 A	0.131 A	0.161 A	1.13 ab	1.04 a	1.15 A ab	1.25 A b	0.03	0.11 A	0.05 A
Glucose-amended	0.168 a	1.118 B c	1.288 B c	0.813 B b	1.16 a	1.43 a	39.33 B c	29.88 B b	0.04 a	13.1 B b	19.9 B c

Upper case letters refer to comparisons among treatments at each sampling time. Lower case letters refer to comparisons among sampling times for each treatment. Values followed by the same lowercase letters denote those not significantly different in each row and by the same upper case letters those not significantly different in each column ($P<0.05$); n.d., not determined.

Table 5. Isolates from glucose-amended microcosms at successive incubation day, their As resistance levels and presence of *Ars* genes.

Isolates and Closest relative (% identity)	Arsenic resistance (mmol l^{-1})		PCR product	
	As(V)	As(III)	*ArsC*	*ArsB*
7 d				
Bacillus sp. 5.1	50	10	+	+
Staphylococcus xylosus 5.2 (100% FJ155342)	25	10	-	+
Bacillus humi 5.3 (100% EU221387)	100	10	+	+
Micromonospora sp. 5.4 (97% FJ214352)	100	5	+	-
Bacillus licheniformis 5.5 (98% FJ607346)	100	5	+	-
Paenibacillus sp. 3.1 (98% AM162319)	50	3	-	-
Bacillus sp. 3.2 (100% FM865886)	50	10	+	-
Rhodococcus ruber 3.3 (100% DQ858963)	100	5	-	-
Bacillus firmus 3.4 (100% EU443751)	100	5	-	-
Bacillus sp. 3.5	50	5	+	-
15 d				
Rhodococcus gordoniae 5.6 (100% EU004418)	100	10	+	-
Bacillus sp. 5.7	100	10	+	+
Bacillus sp. 5.8 (100% FM205065)	100	10	+	-
Bacillus firmus 5.9 (97% FJ613626)	100	10	-	-
Bacillus sp. 3.6	50	5	+	-
Bacillus indicus 3.7 (99% AJ583158)	100	5	-	+
Bacillus sp. 3.8	100	10	+	-
Bacillus sp. 3.9 (100% DQ993311)	25	5	+	-
Bacillus sp. 3.10	50	5	+	-

Fig. 1. Numbers of aerobic (A) and anaerobic (B) heterotrophs in unamended (white) and in glucose-amended microcosms (black) during a 30-d incubation time (n=3). Letters refer to comparison among sampling times for each treatment. Values with the same letters are not significantly different according to Tukey's test ($p \leq 0.05$).

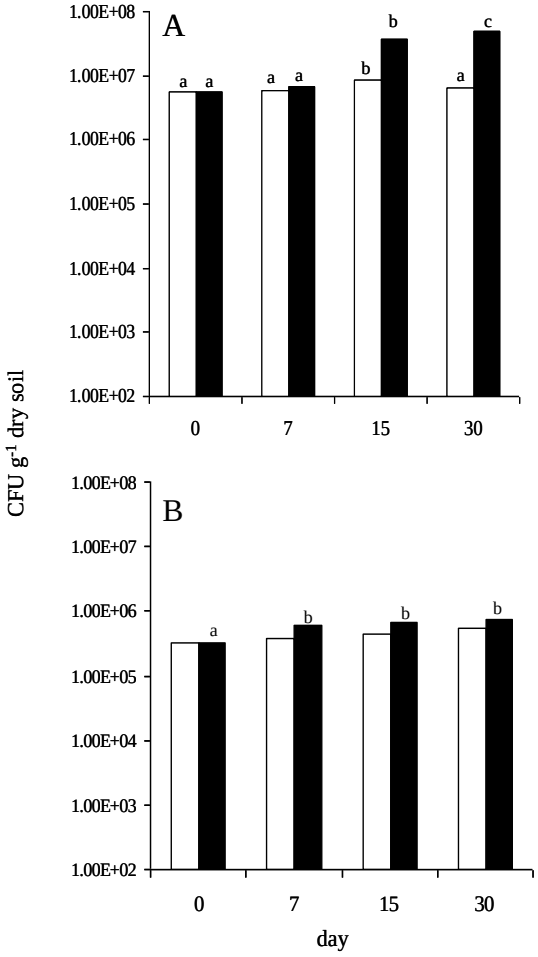


Fig. 2. Time course of pH (A) and redox potential (B) in controls (×), in unamended (○) and in glucose-amended (▲) microcosms during 30-day incubation time (n=3). Values with the same letters in each line are not significantly different according to Tukey's test ($p \leq 0.05$).

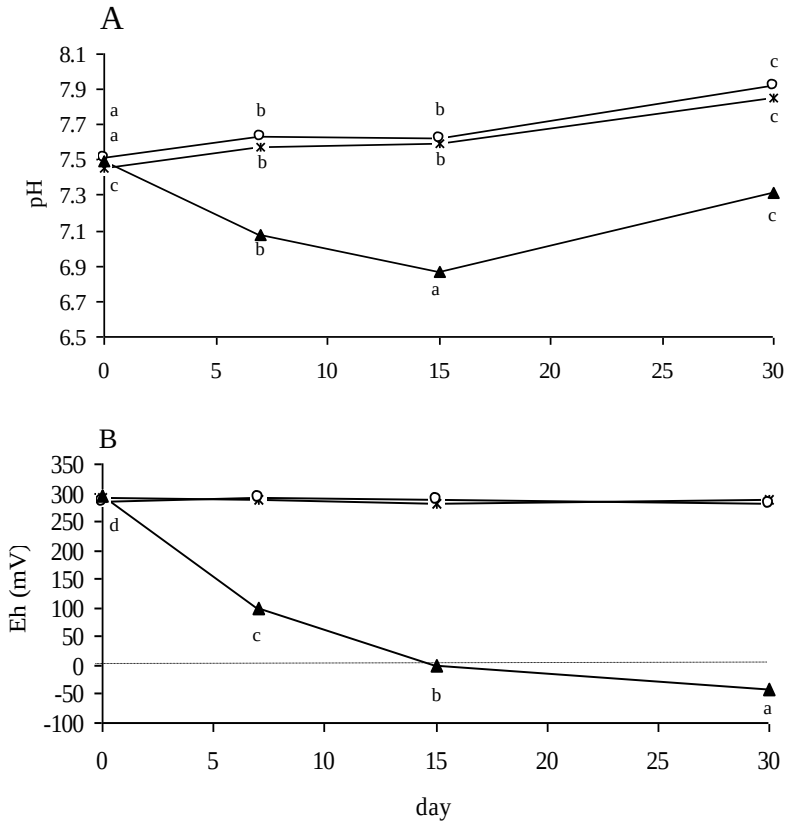


Fig. 3. DGGE profiles of 16S rDNA gene fragments of the bacterial community in unamended (- G) and glucose-amended (+ G) microcosms at successive incubation times. Side letters indicate bands discussed in the result section: (a) Uncultured *Flavobacterium* sp. (AM230410); (b) Uncultured *Flavobacterium* sp. (EU097093); (c) Uncultured *Flavobacterium* (EF378178); (d) Uncultured *Flavobacterium* sp. (EU297120); (e) Uncultured *Paenibacillus* sp. (DQ839177); (f) *Beggiatoa* sp. (L40994).

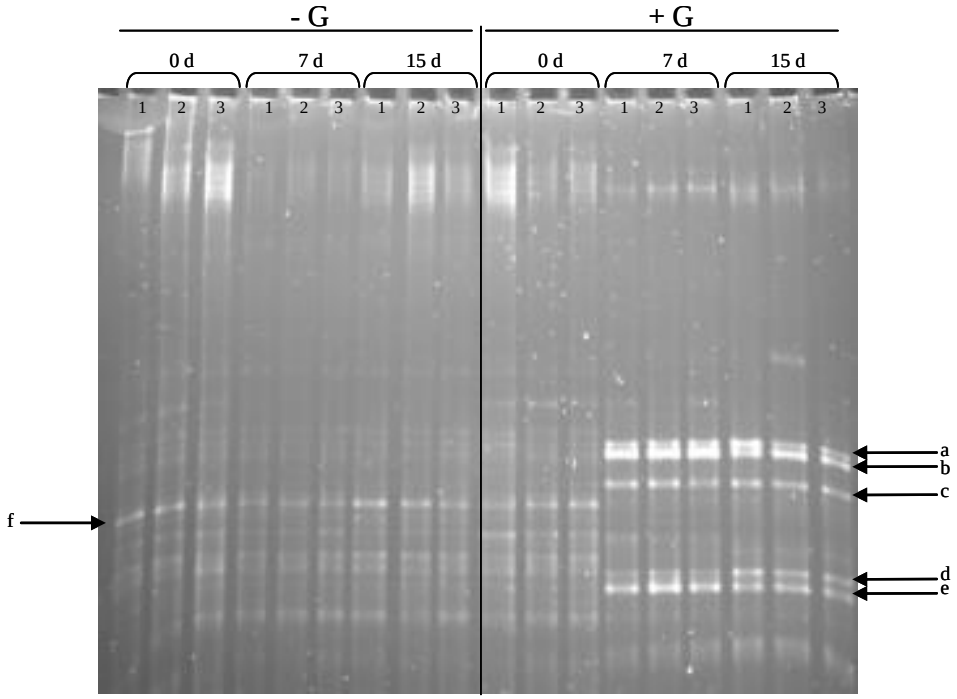


Fig. 4. As speciation in the aqueous phase in glucose-amended microcosms during a 30-day incubation time (n=3). Letters refer to comparison among sampling time for each treatment. Values with the same letters are not significantly different according to Tukey's test ($p \leq 0.05$).

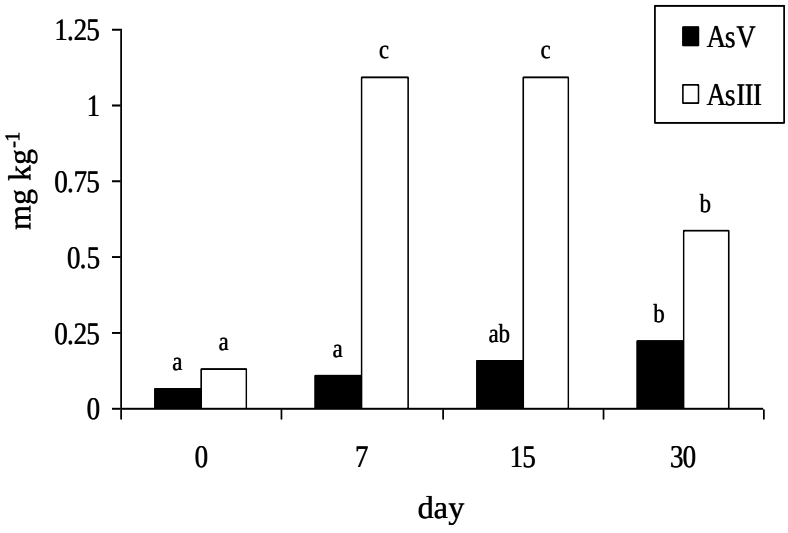
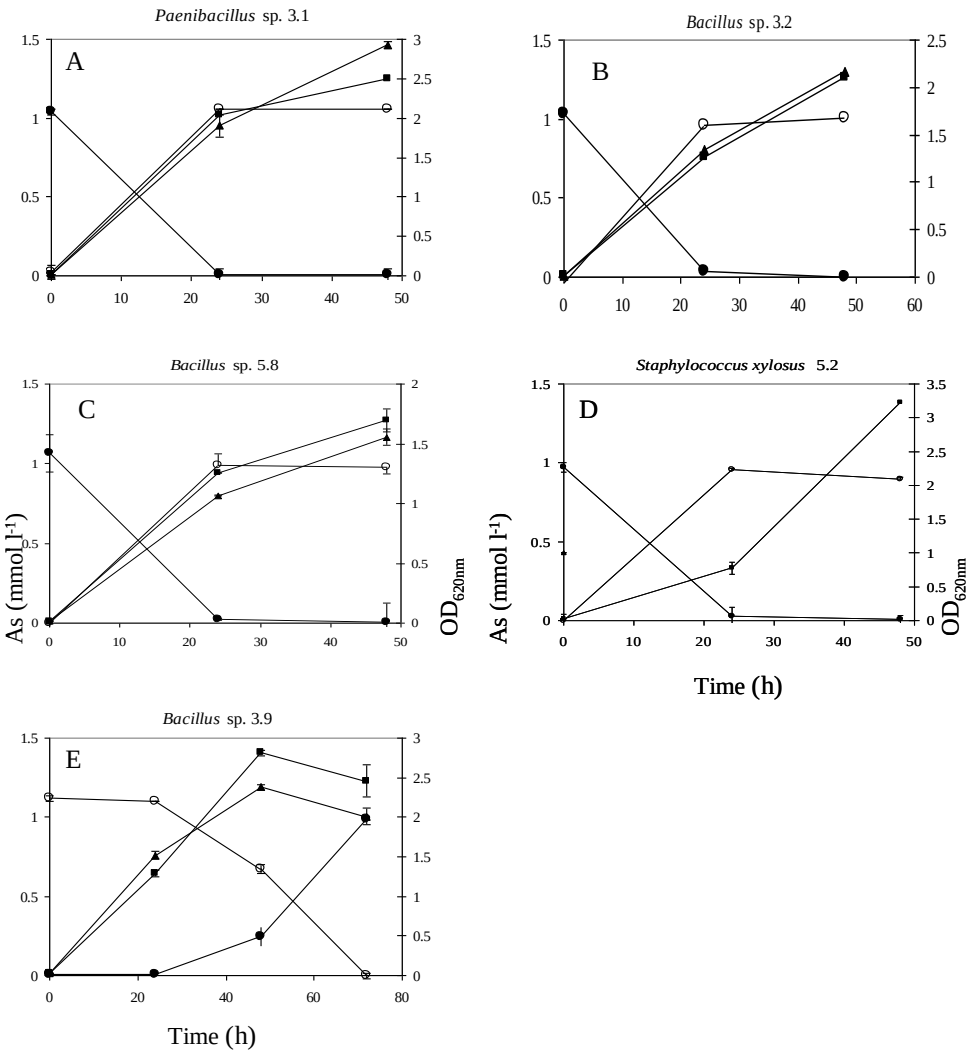


Fig. 5. Bacterial growth (OD_{620nm}) in presence (■) and in absence of As (▲) and corresponding content of As(V) (●) and As(III) (○) by isolates (Mean ± SD; n = 3).



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VI

Influence of Microorganisms on Arsenic Mobilization and Speciation in a Submerged Contaminated Soil: Effects of Citrate.

A. CORSINI, L. CAVALCA, P. ZACCHEO, L. CRIPPA, V. ANDREONI

Influence of Microorganisms on Arsenic Mobilization and Speciation in a Submerged Contaminated Soil: Effects of Citrate.

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Abstract

Batch–type leaching experiments were performed on a multipolluted soil containing pyrite cinders to evaluate the effect of indigenous bacteria on arsenic mobilization. The bacteria, under submerged conditions using citrate as the carbon source, enhanced the solubilization of arsenic that occurred simultaneously with the dissolution of iron and manganese oxides. At the same time a relevant amount of copper was abiotically released. Our results suggested that Fe(III) reduction enhanced the release of As. As(III) prevailed in the first 7 d of incubation at high redox potential (Eh) suggesting a role played by aerobic As-resistant bacteria bearing *arsC* genes for arsenate reduction. As(V) became prevalent in consequence of As liberation from Fe oxides and the lowering of Eh driven by citrate inhibited

the growth and activity of As(V)resistant bacteria. Populations of *Bacillus*, *Pseudomonas*, and *Geobacter* were stimulated by the addition of citrate as evidenced by Denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA genes. Putative *Ars* genes were retrieved in *Bacillus*, and *Pseudomonas* isolates of strains, which were able to reduce 2 mM of arsenate in liquid cultures. These results confirm that arsenic mobilization can be enhanced in an oxygen-depleted soil, particularly with the decomposition of citric acid that is quickly taken up and degraded by soil microorganisms. In addition, this study highlights the complex role of citrate on biotic and abiotic transformation of contaminants in soil. High microbial efficiency in citrate assimilation, interactions among citrate and the soil surfaces and among citrate and dissolved ions can explain this behavior.

Introduction

Nowadays most of the uses of arsenic (As) in agriculture and industry have been discontinued, but residues from such activities have left numerous As-contaminated sites (Leist et al., 2000) that represent a serious health concern worldwide. In soils, As is mainly present as arsenate, As(V) or arsenite, As(III). As(III) is a more mobile and toxic species, while As(V) is preferentially adsorbed on soil compounds like clay, (hydro)oxides of Fe, Mn and Al, and organic matter (Bissen and Frimmel, 2003).

The biogeochemical cycle of As involves several physical and chemical processes (precipitation/solubilization, adsorption/desorption, and redox processes) as well as biological mechanisms, especially those involving bacteria, such as As(V) reduction, As(III) oxidation, and the various methylation reactions (Al-Abed et al., 2007; Lièvremont et al., 2009). Microbial reduction of As(V) can occur through an intracellular mechanism of detoxification (Macur et al., 2001; Bachate et al., 2009; Corsini et al., 2010) or a dissimilatory reduction (Zobrist et al., 2000). The first

mechanism is based on the expression of the *ars* operon and it is functional in aerobic and anaerobic environments. Differently, dissimilatory As(V) reduction, which is carried out by a membrane-bound enzyme and is encoded by the *arr* operon (Macy et al., 2000), usually occurs under anoxic conditions and microorganisms gain energy to grow. Microbial oxidation of As(III) is carried out by heterotrophic and chemoautotrophic bacteria. As(III) oxidation represents a detoxification mechanism for heterotrophic bacteria (Gihring et al., 2001; Silver and Phung 2005) and an energy source for aerobic and nitrate-reducing autotrophic bacteria (Santini et al., 2000; Oremland et al., 2002; Silver and Phung, 2005).

Dissolved natural organic acids at low molecular weight are one of the most labile sources of C in soil and in the rhizosphere. Among these, citric acid, once released into the soil solution, may be taken up by soil microorganisms, mobilize poorly soluble nutrients (P, Fe, Zn) (Kirk et al., 1999), and accelerate mineral weathering. In addition, it may affect As adsorption and desorption especially at acidic pH (Zhang et al., 2005). Arsenic sorbed to particles of iron (hydro)oxides can be easily solubilized in the aqueous phase under different environmental conditions due to microbial activity of both Fe(III)- and As(V)-reducing bacteria. As(V)-reducing bacteria and As(III)-oxidizing bacteria have been found to mediate As transformations in forests, agricultural soils, limed-mine tailings, and in soils containing mineral processing wastes (Macur et al., 2001; Rhine et al., 2008; Bachate et al., 2009; Corsini et al., 2010), and also in more reduced environments such as groundwater aquifer, and sediments (Kulp et al., 2007; Pederick et al., 2007; Song et al., 2009).

The objectives of this work were: (i) to evaluate the potential mobility of As, Fe, Mn, and Cu from a multipolluted soil; (ii) to verify if As reduction may be coupled to Fe oxides reduction; and (iii) to examine the effect of indigenous bacterial activity on As, Mn, and Fe solubilization in a submerged experimental system

spiked with citrate, used as a model of labile C source of thee soil, and (iv) to study the dynamics of the microbial community related to As mobilization.

Materials and Methods

Sampling and site description

The soil polluted with As and heavy metals was collected from a site located in the Northeast of Italy (Udine). The site was contaminated by pyrite cinders, a by-product of sulphuric acid manufacturing operations (800°C). The cinders were buried into the soil and covered by a layer of soil for about 40 years. In 2005, the polluted soil was obtained by mixing the covering soil and the cinders in equal amount (v/v). A composite sample (50 kg) of the polluted soil, whose characteristics are shown in Table 1, was collected, sieved, and stored at 4 °C until use in submerged microcosm leaching experiments.

Set up of microcosms.

To examine the effect of the bacterial activity on As, Mn, Fe, and Cu mobilization from the polluted soil, 50 g of soil was mixed with 50 g of water in 100-ml tubes. Three sets of tubes (microcosms) were prepared: one amended with citrate (0.23 %, w/w) as carbon source (citrate-amended); one left without citrate (unamended), and one amended with citrate and previously treated for 16 h with formaldehyde (0.04%, w/v) to inhibit the microbial activity (Tuominen et al., 1994) (citrate-controls). The treatment with formaldehyde resulted in a ten-fold reduction of the number of heterotrophs. $Ca(NO_3)_2$ (7.83 mg) was added to adjust the C/N ratio of citrate-amended microcosms to 30. The same amount of $Ca(NO_3)_2$ was also added to the unamended microcosms. The tubes were closed with cotton plugs and

incubated statically at 25°C. Three sacrificial replicates of each microcosm were prepared for each sampling time. Time courses of pH, redox potential (Eh), total As, As(V), As(III), Fe, Mn, and Cu contents in the aqueous phase were determined on successive incubation days.

The number of aerobic heterotrophs was determined in all the microcosms by conventional plating technique on 1/10 strength Tryptic Soy Agar (TSA/10). Plates were incubated at 28°C for 10 days and then the UFCs counted.

Chemical determinations

Redox potential and pH in microcosms were measured in the upper aqueous phase of the tubes, retaining the electrodes until the measurements were stabilized. The tubes were successively shaken for 10 min and then centrifuged at 6000 rev per min^{-1} for 15 min. The supernatant was collected, filtered (0.45 μm), and stored at -20°C until the determination was completed. As, Fe, and Mn contents in the solution were determined in 10 ml of supernatant after acidification with HNO_3 to pH 2.0. For speciation of arsenic, 10 ml of un-acidified supernatant were passed through a WATERS Sep-Pak_ Plus Acell Plus QMA cartridge (Waters, MA) according to Corsini et al. (2010). Fe, Mn, Cu, and As contents were determined by ICP-MS (Varian, Victoria, Australia). Standards of Cu, Fe, and Mn ranging from 0 to 1 mg l^{-1} were prepared from multistandard solution (Varian Victoria, Australia); standards of As from sodium arsenite ($NaAsO_2$) (Sigma-Aldrich).

Denaturing gradient gel electrophoresis (DGGE) analysis

Dynamics of the bacterial communities of citrate amended and unamended microcosms related to the addition of citrate was studied at successive incubation times in triplicate samples with DGGE applied to the hyper-variable region V3 of

bacterial 16S rRNA gene. PCR amplification of V3 regions was carried out using a nested PCR approach as described in Corsini et al. (2010) by using primer pair P341f-GC clamped and P534r (Muyzer et al. 1993). PCR products were loaded onto polyacrylamide gels (8%) in a DCODE Universal Mutation Detection System (Biorad) apparatus. The linear denaturing gradient of urea and formamide ranged from 40% (top) to 60% (bottom) where 100% denaturant gels contained 7 M urea and 40% formamide. DGGE bands of interest were excised with a sterile scalpel, suspended in sterile Milli-Q water, and used as template for V3 amplification by primers V3 forward without GC clamp and V3 reverse.

Isolation and characterization of aerobic As-resistant bacteria

Appropriate serial dilutions of soil suspensions from citrate-amended microcosms at 7 days of incubation were plated on TSA/10 medium containing 5 mmol l^{-1} of As(III). After 10 days of incubation at 28°C in aerobic conditions, 10 colonies were randomly isolated from the plates. Single colonies were streaked to purity on the same medium. Strains were maintained in glycerol stocks at -70°C.

Resistance to arsenic and to copper was determined by growing the isolates in flasks containing 20 ml of TMM medium (Mergeay et al., 1985) supplemented with 0.6% (w/v) gluconate (TMMG) and increasing concentrations of either As(V) ($Na_2HAsO_4 \cdot 7H_2O$, from 0 to 100 mmol l^{-1}) or As(III) ($NaAsO_2$, from 0 to 50 mmol l^{-1}) or Cu(II) ($CuCl_2$, from 0 to 1 mmol l^{-1}). The flasks were incubated at 30°C and kept shaken at 180 rev min^{-1}, and checked for growth after 72 h of incubation. For the oxidation of As(III) or reduction of As(V), each strain was inoculated into 3 vials, each containing 20 ml of TMMG supplemented with 2 mmol l^{-1} As(III) or As(V). Three vials with no As were inoculated to compare the growth of the microorganisms in the absence of As(III) or As(V) and three vials were also prepared without inoculum to check for abiotic transformation of As. At each

sampling time, 2 ml of cell suspensions were removed to follow cell growth by measuring the OD_{620nm} and to determine As(V) and As(III) concentrations by spectrophotometric analysis (Corsini et al., 2010). Strains were identified by sequence analysis of 16S rRNA gene.

Arsenic resistance gene amplification in soil and isolates

PCR amplification of As-resistance genes for As(V) reductase (*ArsC*) and for different As(III) efflux pumps (*ArsB, ACR3(1)* and *ACR3(2)*) were conducted as previously described (Cavalca et al., 2010).

Sequence analysis

16S rDNA, arsenic resistance genes and DGGE DNA bands were sequenced using the *Taq* Dye-Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, USA) with the respective primers. The forward- and the reverse samples were run on a 310A sequence analyzer (Applied Biosystems, USA). Sequences were compared with the entire GenBank/EMBL nucleotide and amino acid databases using the BlastN and BlastX query programs (http://www.ebi.ac.uk/Tools/blastall/index.html) and EzTaxon server 2.1 (Chun et al., 2007) was used to attribute type strain species to newly isolated strains and to DGGE bands.

Sequences were deposited under the accession numbers: FN825804, FN825805 and FN825806 for arsenic resistance genes and from FN825781 to FN825785 for 16S rRNA genes.

Statistical analysis. Analytical data from the microcosm experiments were compared applying ANOVA and the b-Tukey test using SPSS version 17.0 (Inc., Chicago).

Results and Discussion

Effect of citrate on pH, redox potential, As, Fe, Cu, and Mn solubilization

The polluted soil under study is a two-component material in which arsenic, iron and copper come predominantly from pyrite cinders, while manganese from the covering soil. Cu, Zn, Pb, and As contents of the polluted soil (Table 1) exceed the threshold values of the Italian legislation (Decreto Legislativo 152/2006). The more labile fractions of the two major contaminants account only for 7.6% and 15% of total As and Cu, respectively.

The addition of citric acid to obtain a final concentration of 1.2 mM comparable to the level found in soil solutions (Zhang et al., 2005), lowered the pH to 6.2 making the soil weakly acidic. However, in 1 d the pH of citrate-amended microcosms and citrate-controls turned to neutrality because of the soil-buffering capacity (Fig. 1). In the successive days, the microbial activity caused a slight and transient acidification of citrate-amended and unamended microcosms.

In citrate-amended microcosms, aerobic heterotrophic bacteria significantly increased, passing in 7 d from 4.5×10^6 to 2.7×10^7 CFU g dry soil^{-1}, while in unamended microcosms and in citrate-controls, they did not increase during the course of incubation. The microbial utilization of citrate and the consequent oxygen depletion drove the citrate-amended microcosms toward anaerobic conditions, as evidenced by the Eh values (Fig. 1) that dropped to -50 mV in 30 days. The decomposition of citrate in soil can be a rapid process that occur with different kinetics dependent on the type of soil mineral phases; however, iron oxides was shown to strongly interact with citrate inhibiting its degradation (Jones and Edwards, 1998).

The rise of Eh to 90 mV in citrate-amended microcosms at d 45 could be ascribable to the oxygen entered into the microcosm that, once dissolved in the aqueous phase

was no more consumed by microorganism for the lack of a promptly utilizable C source. Without the addition of citrate, the oxygen consumption by microorganisms was obviously too small to induce a decrease in Eh potential.

The significant release of As, Fe, and Mn into the aqueous phases observed up to 15 days in citrate-amended microcosms indicated the biological nature of the processes. In particular, strong mobilization of As was observed from d 3 to d 15 (Fig. 1). As(III) was the main species in the leaching solution (Fig. 2) when oxic conditions prevailed (Eh > 250 mV). The progressive As(III) appearance up to d 15 could be due to the detoxifying activity of As-resistant bacteria with *Ars* genes and/or to arsenate respiring bacteria with *Arr* genes. The strong release of As(V) observed at d 15 was probably related to the dissolution of iron oxides present in the soil. As(V) was no more reduced even if moderately reducing condition were established. Aerobic bacteria that reduce As(V) through the *ars* operon could be inhibited by the redox conditions and anaerobic respiring bacteria could be absent or not induced by citrate. As(V) can also be observed under reducing conditions due to the slow kinetics of As(V) transformation (Masscheleyn et al., 1991). Re-precipitation or re-adsorption of about 30% of dissolved As occurred from d 21.

No As solubilization were detected in unamended microcosms. In citrate-controls, citrate induced an immediate release of small amount of As(V) through direct and/or indirect effect (Fig. 2). However As(V) was re-immobilized at d 15 when the pH of the soil approached neutrality.

In citrate-amended microcosms, biotic and abiotic processes concurred to Fe solubilization which occurred with two different kinetics. In the first 7 days, 20 mg kg^{-1} Fe were released for the acidification induced by the addition of citric acid. At d 15, the Eh (75 mV) was permissive to the growth and activity of some indigenous anaerobic bacteria that could reduce Fe(III) leading to the dissolution of large amount of iron oxides (Fig. 1). In citrate-controls Fe, abiotically solubilized, was maintained into solution by the citrate-chelating capacity.

The biological iron oxides dissolution was concomitant to a great increase of total soluble As content (Fig. 1), probably for the release of As occluded into Fe oxides (Weber et al., 2010). Differently, decoupling between Fe and As release was previously observed to occur when the same soil was spiked with glucose (Corsini et al., 2010). By adding glucose on the same C basis of citrate, As mobilization occurred first and that of Fe followed, in accordance with the results of Van Geen et al. (2004) and Radloff et al.(2007). Moreover, we found that glucose showed the lowest As mobilizing capability (1.28 mg As kg^{-1} vs 2.64 mg As kg^{-1}). Opposite decoupling trend was also found in some natural and artificial sediments (Islam et al., 2004; Solaiman et al., 2009).

Similarly to Fe mobilization, biotic and biotic processes were involved in Mn dissolution (Fig. 1). In citrate-amended microcosms, the microbial dissolution of Mn oxides occurred at oxic conditions (250 – 300 mV), became evident at d 7 and preceded Fe oxide solubilization. In citrate-amended microcosms, Mn was almost entirely re-immobilized at d 21 while in citrate controls it remained into solution for the citrate-chelating capacity. The concomitant re-immobilization of Mn and As suggested the formation of a solid phase consisting of $Mn_3(AsO_4)_2$ (Masscheleyn et al., 1991).

Finally, high amounts of copper were also solubilized (Fig. 1) imparting a blue coloring to the aqueous phases of citrate-amended and citrate-control microcosms. Cu was completely re- immobilized in citrate-amended microcosms and partially in citrate-controls.

Effect of citrate on soil bacterial community

Citrate addition gave rise to noticeable modifications of total soil bacterial community of citrate-amended microcosms, as evidenced by the increment of four bands (1–4) since d 3 and four bands (5–8) since d 15 in the DGGE profiles (Fig.

3). Bands 1 and 4 (99.3% identity of nucleotide sequence to *Pseudomonas vranovensis* CCM 7279(T) Acc. Num. AY970951) were visible until d 7, but they disappeared afterward. With regards to bands 2, 3 and 5, the first two co-migrating into the unique band 5 in gel "b", belonged to *Pseudomonas frederiksbergensis* (100% identity of nucleotide sequence to Acc. Num. AJ249382) and they dominated the profile until the end of the experiment. Bands 6, 7 and 8 were evident at d 15 of incubation; only band 7 persisted at longer incubation time. They belonged to *Bacillus cereus* (100% identity of nucleotide sequence to Acc. Num. AE016877), to *Geobacter bemidjiensis* (93.2% identity of nucleotide sequence to Acc. Num. CP001124) and to *Clostridium nitrophenolicum* (100% identity of nucleotide sequence to Acc. Num. AM261414). The DGGE profiles of total soil bacteria in unamended microcosms did not alter during the course of the experiment. The bacterial community of citrate-controls was not analyzed due to the absence of bacterial growth and to modifications of As, Fe, Cu and Mn content ascribable to the chelating activity of citrate.

Geobacter species, previously considered strict anaerobes, survive in oxic subsurface environments and rapidly become the predominant Fe(III)-reducing microorganisms in a diversity of subsurface sediments containing organic electron donors in order to stimulate anaerobic dissimilatory Fe reduction (Snoeyenbos-West et al., 2000). Dissimilatory Fe(III)-reduction of *Geobacter* strains identified in soil DNA at d 15 of incubation, could have played a role in the release of As from Fe oxides in the presence of citrate. Recently, Prakash et al. (2010) characterized *Geobacter daltonii* strain FRC-32^{T} gaining energy from the respiration of Fe(III)-oxyhydroxide, soluble ferric iron, elemental sulfur and Mn(IV) coupled with the oxidation of small organic acids. The role of *Geobacter* in As(V) dissimilatory reduction has up to day been excluded (Islam et al., 2005).

The analysis of DNA of unamended and citrate-amended soils gave evidence of the existence of a bacterial As-resistant population carrying *ArsC* and *ArsB* genes but

not of *ArrA* gene. In citrate-amended microcosms at d 15, besides the retrieval of these genes, *ACR3(1)* gene was successfully amplified, indicating that addition of citrate had possibly enriched the soil of microorganisms with this As(III) efflux system. Firmicutes often carry *ACR3(1)*, counting for the 42% of the total *ACR3* sequences deposited in GenBank. The amplification of *ACR3* at d 15 in citrate-amended microcosms was mirrored by the intensification of *Bacillus* and *Clostridium* DGGE bands, indicating that these microorganisms were possibly involved in the reduction of As(V) via intracellular detoxification mechanisms without excluding a participation in an As(V) respiration process. Respiration of As(V) is well-documented for *Clostridium* and *Bacillus* strains (Stolz et al., 2006).

Identification of aerobic As-resistant isolates and As transformation patterns

Arsenic-resistant bacteria were isolated from citrate-amended microcosms at d 15 of incubation (Table 2). They belonged to *Bacillus cereus*, *Pseudomonas azotoformans*, and *Rhodococcus erythropolis* and they were resistant to high concentration of arsenate and arsenite. In particular, *Bacillus cereus* AC4 and *Rhodococcus erythropolis* AC5, which had the highest arsenite and arsenate resistance were also able to grow on TMMG medium in the presence of 0.5 mmol $l^{-1}$of Cu(II). In *Bacillus cereus* strains AC2 and AC4 *ArsC* genes were amplified with a deduced aminoacid sequence identities of 100% and 92%, respectively, to arsenate reductase of *B. cereus* strains deposited in GenBank; *ArsB* gene for arsenite efflux pump of *Pseudomonas azotoformans* AC3, had 85% of identity to arsenical pump membrane protein of *Pseudomonas syringae* pv. *syringae* strain B728a. The same *Bacillus cereus* strain was retrieved by DGGE band analysis and strain isolation from citrate-amended microcosms and a good correspondence was also found at the genus level in the retrieval of different species of *Pseudomonas.* An involvement in As detoxification processes could be ascribable to these

populations within the bacterial community of citrate-amended microcosms. DGGE bands of *Geobacter* and *Clostridium* were not mirrored by the isolation of such strains, probably for the lack of appropriate electron acceptors or growth conditions.

Bacillus cereus AC2 and AC4, *Pseudomonas azotoformans* AC3 and *Rhodococcus erythropolis* AC5 reduced completely 2 mmol l^{-1} of As(V) in 24 h under aerobic conditions, *Rhodococcus erythropolis* AC6 in 48 h (Fig. 4). None of them was able to oxidize As(III). Arsenic transformations did not occur in controls with no cells, indicating that the processes were due to microbial activities.

Conclusions

Our results indicate that the mobilization of inorganic contaminants during flooding of polluted soils depends on several factors such as the nature of dissolved organic substances, the presence of redox active minerals (Fe and Mn oxides) and of the native microflora. Oxic soils, contaminated by As and rich in Fe oxides, when flooded and in presence of a labile C source can liberate As bound to oxides by microbial Fe(III) reduction. In our study, the great abundance of Fe oxides did not hamper citrate biodegradation, differently from the findings of Jones and Edwards (1998). Only a negligible solubilization of total Fe (0.02 %) and As (0.6%) was detected within 45 d of submersion. In addition, our results, highlight the wide interactions among citrate, and the dissolved ions occurring in a soil. In particular, citric acid modifies the solubility of copper thus potentially increasing the bioavailability of inorganic pollutants of environmental concern. Due to their high degree of reactivity, Mn oxides can play a role in As(V) re-immobilization, contributing in the control of the soluble As content of the soil. The solubilization of As is of environmental relevance because of the possibility that bacteria can reduce As(V) to As(III). Considering the greater mobility of As(III), it is therefore

important to minimize the ecological risks connected with As solubilization in case of the submersion of soils rich in dissolved organic substances.

Acknowledgments

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Table 1. Physical and chemical characteristics of the polluted soil (Corsini et al., 2010)

pH_W		7.75
Organic carbon (g kg^{-1})		9.14
C.E.C. ($cmol^{(+)}$ kg^{-1})		2.34
Olsen P (mg kg^{-1})		7.78
Exchangeable K (mg kg^{-1})		132
Gravel (%)		33
Sand (%)	2 - 0.05 mm	72.4
Silt (%)	0.05-0.002 mm	25.6
Clay (%)	< 0.002 mm	2.0
Total Fe*		290
Total Mn		165
Total Cu		1527
Total Zn		980
Total Pb		255
Total As		446
As sorbed		34
As bound to oxides of Fe and Al		190
As in residual phase		224

* Fe concentration is in g kg^{-1}

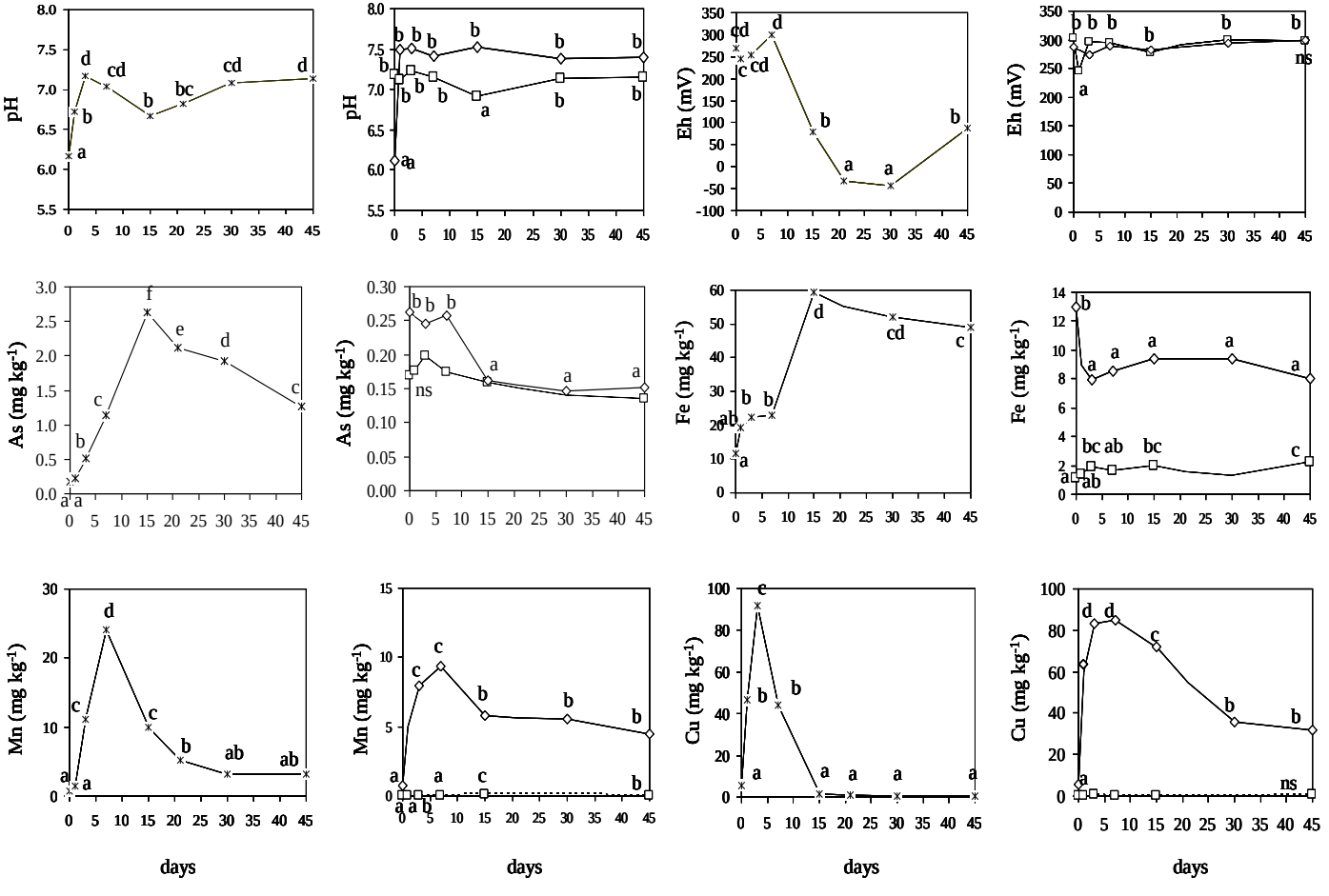
Table 2. Characterization of isolates from citrate-amended microcosms, their As and Cu resistance levels, and As-resistant genes.

Isolates and closest relative (% identity)	As resistance (mmol l^{-1})		Cu (II) resistance (mmol l^{-1})	PCR product	
	As(V)	As(III)		*ArsC*	*ArsB*
Bacillus cereus AC2 (100% AE016877)	20	5	0	+	-
Pseudomonas azotoformans AC3 (99.9% D84009)	100	10	0	-	+
Bacillus cereus AC4 (99.9% AE016877)	100	30	0.5	+	-
Rhodococcus erythropolis AC5 (100% AP008957)	100	50	0.5	-	-
Rhodococcus erythropolis AC6 (99.7% AP008957)	20	10	0	-	-

-, not detectable with the primer used

Figure 1. Time course of redox potential and element solubilization in citrate-amended (✳), in citrate-controls (◊) and in unamended (□) microcosms during a 45-day experiment (n=3). Letters refer to comparison among sampling times for each treatment. Values with the same letters are not significantly different according to Tukey's test ($p \leq 0.05$).

Fig. 1.



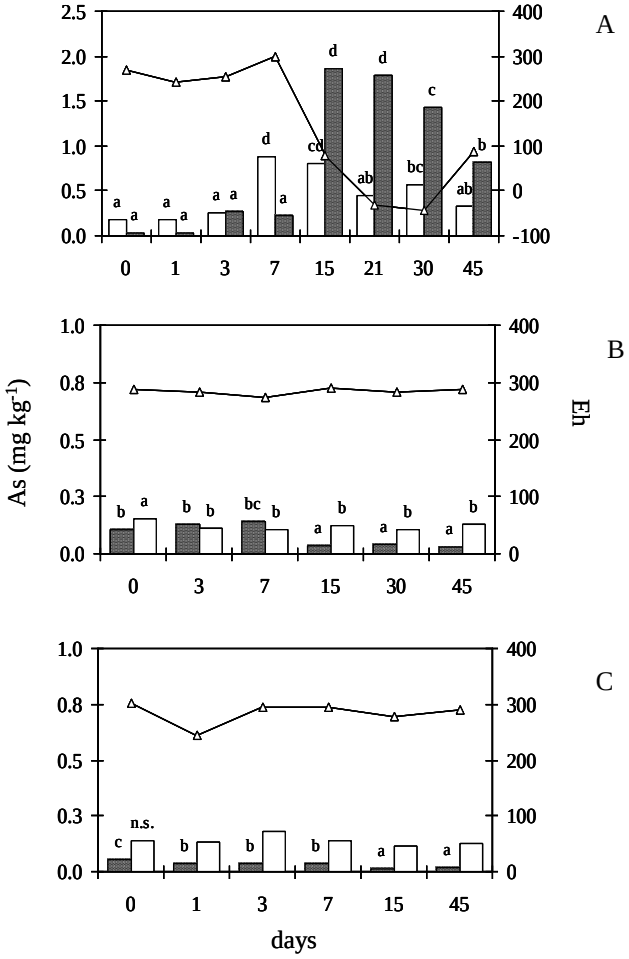


Figure 2. Dynamics of As forms and Eh in citrate-amended microcosms (A), citrate-controls (B) and unamended microcosms (C) during a 45-day experiment (n=3); As(V) black bars; As(III) white bars; Eh (Δ). Letters refer to comparison among sampling times for each As form. Values with the same letters are not significantly different according to Tukey's test ($p \leq 0.05$).

Figure 3. DGGE profiles of 16S rRNA gene fragments of the bacterial community in unamended and citrate-amended microcosms at successive incubation times (a, 0–7 days; b, 15–45 days) in triplicate samples. M, marker composed of DNA bands of *Pseudomonas azotoformans* AC3 (a), *Bacillus cereus* AC2 and AC4 (b, c). Side numbers indicate bands discussed in the result section: *Pseudomonas vranovensis* (1, 4); *Pseudomonas frederiksbergensis* (2, 3, 5); *Bacillus cereus* (6); *Geobacter bemidjiensis* (7); *Clostridium nitrophenolicum* (8).

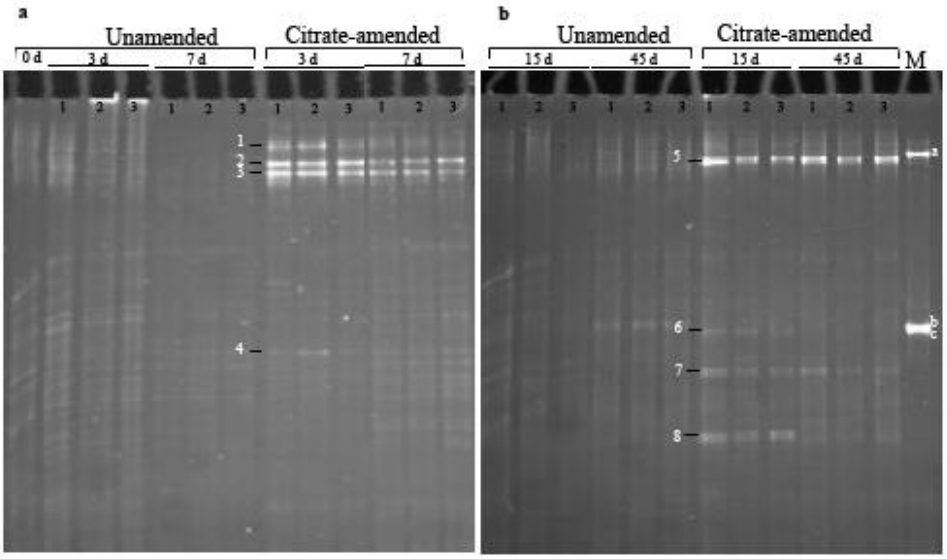
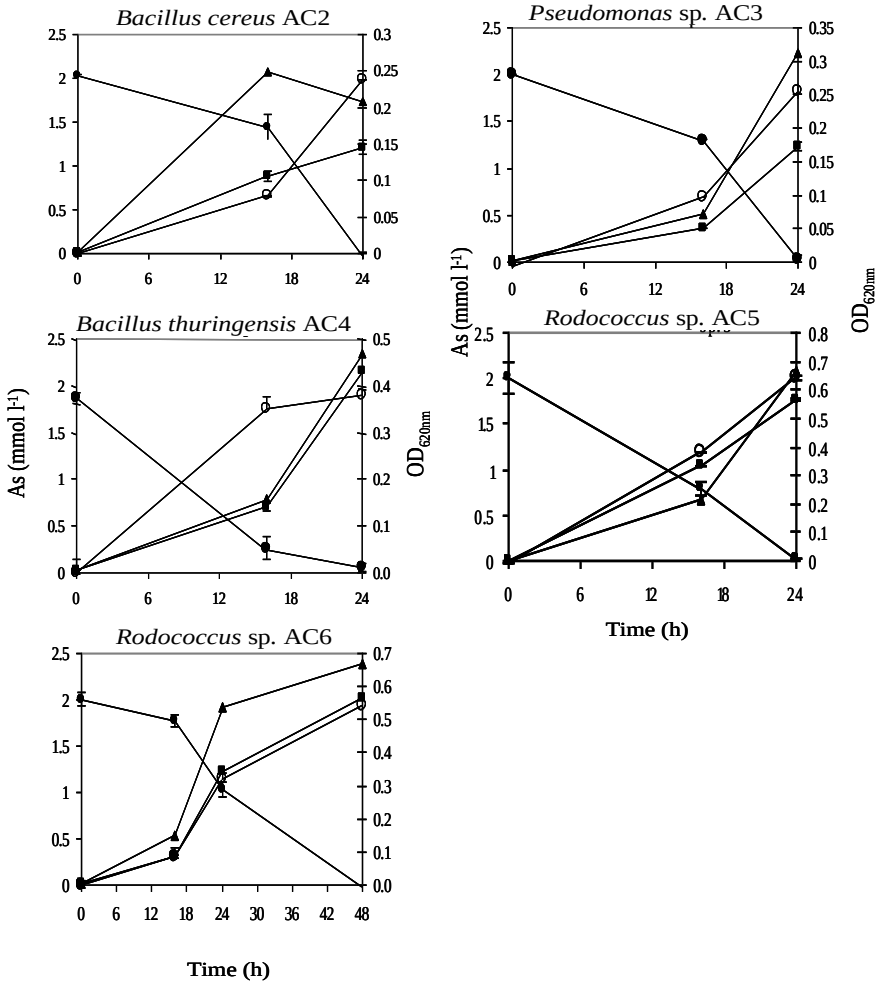


Figure 4. Bacterial growth (OD_{620nm}) of isolates in presence (■) and in absence of arsenate (▲) and corresponding content of As(V) (●) and As(III) (○) (Mean ± SD; n = 3). The OD values reported are 1:5 dilution of the samples.



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VII

Dynamic changes in *arsC*, and *arrA* gene copy numbers during As reduction in contaminated soil under different environmental conditions

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Dynamic changes in *arsC*, and *arrA* gene copy numbers during As reduction in contaminated soil under different environmental conditions

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Introduction

Arsenic (As) is an ubiquitous contaminant associated with natural weathering of rocks and anthropogenic inputs associated with mining and agricultural applications. In oxic environments, such as surface sediments and soils, arsenate, As(V), is dominant, but under reducing conditions it is converted to arsenite, As(III) (Masscheleyn et al., 1991). Because of differences in relative sorption strengths of As(III) and As(V), changes in soil redox conditions have been found to alter aqueous As concentrations (Ackermann et al., 2008). Microorganisms involved in As(V) reduction can be As-resistant or As-respiring organisms (Murkhopadhay et al., 2002). The former can reduce As(V) in oxic environments, through a detoxification mechanism, based on the expression of the ars operon (Silver and Phung, 2005). Alternatively, dissimilatory As(V) reduction is carried out by a membrane-bound enzyme, encoded by the arr operon and it usually occurs under anoxic conditions (Macy et al., 2000). Moreover, As(V)-respiring bacteria utilize As(V) as a terminal electron acceptor while oxidizing various organic

(lactate, acetate, formate and aromatic compounds) or inorganic electron donors (Oremland and Stolz, 2005; Pérez-Jiménez et al., 2005; Hollibaugh et al., 2006; Lear et al., 2007). Many As(V)-reducing bacteria, possessing *arsC* or *arrA* genes, have been isolated from various environments, such as agricultural soils, forests, soils containing mineral processing wastes, soda lakes, groundwater aquifer, and sediments (Macur et al., 2001; Saltikov et al., 2003; Rhine et al., 2008; Song et al., 2009; Cavalca et al., 2010). The *ars* operon as well as the *arr* operon appear to be relatively widespread throughout microorganisms (Jackson and Dugas, 2003; Páez-Espino et al., 2009).

Quantitative real-time PCR (qPCR) provides an useful tool to quantify functional genes and to monitor their fluctuations in various ecosystems (Hai et al., 2009; Weber and King, 2010). Moreover, functional genes have been successfully targeted in bioremediation studies to investigate microbial populations responsible for the degradation of various contaminants, such as aromatic hydrocarbon or herbicides. (Cèbron et al., 2009; Paulin et al., 2010; Rodríguez-Cruz et al., 2010)

Although many studies have demonstrated the application of qPCR-based techniques in the analysis of contaminated matrices, not much data are available on abundances of functional genes involved in the arsenic cycle. Recently Quéméneur et al. (2010) demonstrated the feasibility of using qPCR and DGGE of *aoxB* genes for monitoring the structure, diversity and abundance of As(III)-oxidizing bacteria in As-contaminated waters. Similarly, Sun et al. (2004) developed a qPCR method for the quantification of the *arsC* gene in As-rich environment in order to evaluate the relative mobility of arsenic. This study only considered the aerobic bacteria involved in the As cycle and consequently only the detoxification mechanisms.

In previous works on As solubilization from a polluted soil under submersion we have reported that the addition of different C sources stimulated the microbial As(V) reduction under reducing conditions (Corsini et al., 2010 and submitted). However, from the DGGE analysis and the isolation of As-resistant strains from

this soil, we did not obtain evidences of the presence of As(V)-respiring bacteria potentially involved in As(V) reduction. However, since As(V) reduction can occur under reducing conditions due to As(V)-respiring bacteria (Lear et al., 2007), we can not exclude their contribute in the As release.

The specific objectives of this paper are: (1) to investigated the occurrence of different microbial pathways for arsenate reduction in the submerged soil, amended with different carbon sources, by quantifying the two functional genes involved in As solubilization, *arsC* and *arrA*; (2) to assess the effectiveness of the *arsC* and *arrA* to be a reliable molecular marker for mobilization of As.

Material and Methods

Microcosm preparation and samplings

The soil polluted with As and heavy metals, collected from a site located in the Northeast of Italy (Torviscosa, Udine) was used to set up flooded microcosms to which glucose or citrate were added to study the influence of the C source on As solubilization.

Soil characteristics and details of the set-up of microcosms are reported in Corsini et al. (2010). At successive incubation times, soil from glucose-amended, citrate-amended was analyzed for the presence of *arsC* and *arrA* genes. Similarly, soil from unamended microcosms (controls) was collected and analyzed. Total DNA was extracted from soil microcosms using a bead-beating method (MOBIO, USA) according to manufacturer's instructions. Particularly, three sacrificial replicates for each sampling times from glucose-amended, citrate-amended and controls were used. Data of redox potential (Eh) and arsenic species for each treatment and sampling time, obtained from Corsini et al. (2010 and submitted), were used to discuss molecular data reported in the present paper.

Primer selection

DNA extracts from the soil microcosms at all sampling time were previously analyzed by PCR for the presence of As(V) reductase (a*rsC*) and As(V) respiratory reductase (*arrA*) during the incubation time. Primer P52f (5'-AGCCAAATGGCAGAAGC-3') and P323r (5-GCTGGRTCRTCAAATCCCCA-3) were used for As(V) reductase *arsC* amplification (Bachate et al., 2009). Malasarn et al (2004) designed degenerate primers ArrAfwd (5'-AAGGTGTATGGAATAAAGCGTTTgtbgghgaytt-3') and ArrArev (5'–CCTGTGATTTCAGGTGCCcaytyvggngt-3') that were used in this study to amplify As(V) respiratory reductase. Primer were checked with software Primer3 for thermostability in order to estimate their suitability in Real time PCR reaction. PCR products were run on 1.5% agarose gels to check the presence of single PCR fragments of the expected size for each gene. Positive PCR products obtained by the *arsC* and *arrA* primers were used for cloning reactions. Briefly, PCR were performed by using the *arrA* and *arsC* primers and reaction mixtures were prepared in a final volume of 25 µl containing 4 µl of 5X Phusion GC buffer (Finnzymes, Helsinki, Finland), 800 µM dNTPs (Fermentas, Burlington, Canada), 1.75 mM $MgCl_2$, 0.4 mM of each primer, 2 U of Phusion DNA polymerase (Finnzymes) and 2 µl of template DNA. The thermal cycling program for both pirmer pairs was as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 30 seconds, 55°C for 1 min, and 72°C for 1 min. PCR products were visualized by UV fluorescence following electrophoresis in a 1.5% agarose gel, and fragments corresponding to *arsC* and *arrA* were excised and purified with the Qiaquick gel extraction kit according to the manufacturer's protocol (Qiagen, Crawley, United Kingdom).

Quantitative real-time PCR

All qPCRs were carried out with Mx3000P qPCR system (Stratagene, La Jolla, CA) using the Brilliant SYBR Green QPCR Master Mix (Stratagene). The reaction mixtures contained 12.5 µl 2X Brilliant QPCR Master Mix (including SYBR green 1 dye and PCR buffer, deoxynucleoside triphosphates [dNTPs], SureStart Taq DNA polymerase, and 5 mM $MgCl_2$); 500 nM of each primer; 2 µl of DNA and DNase/RNase-free water (Sigma-Aldrich) for a final volume of 25 µl. The qPCR conditions were the same for all primer sets and consisted of an initial 10-min incubation at 95°C for enzyme activation, followed by 40 cycles of denaturation at 95°C (30 s), annealing at 55°C (1 min), and extension at 72°C (1 min). At the end of the qPCR a dissociation curve with melting temperature (Tm) of the amplified product was generated. The conditions were as follows: a cycle of 95°C for 1 min and 55°C for 30 s, finally increasing to 95°C by increments of 0.5°C s^{-1}, each followed by a fluorescence acquisition step. These curves were used to ensure PCR product specificity by observation of a single melting peak, and results were further confirmed by the presence of single PCR fragments of the expected size in 1.5% agarose gel.

Standard curves

To test possible inhibitory effects on quantitative PCR amplification caused by soil, the optimal dilution for soil DNA extract was determined by pre-experiments. Particularly, the efficiency of the standard curves generated for each gene in the presence of soil was compared with that of the standard curve obtained with plasmid and pure strain only. Dilution series of plasmid DNA, targeting *arrA* of *Desulfitobacterium hafniense* DCB-2 (Acc Num CP001336, Homology 82%) and of *Bacillus* sp. 3.2 (FM865886), strain isolated from the soil under study and

harbouring *arsC* gene (Corsini et al., 2010) were used to generate the standard curves. Triplicate sets of standards and no template controls (NTC) were run in parallel with all samples. The amplification efficiencies (E) were calculated from the slope of the standard curve using the equation $E = [10^{(-1/slope)} -1]$. The resulting standard curves were used to quantify the number of *arrA* and *arsC* copies in the samples, basing on the assumption that only one copy of the target gene is present per genome.

Cloning and Sequencing of ArsC and arrA genes

Cleaned PCR products were ligated into the pCR2.1-TOPO vector and transformed into TOP10 chemically competent *Escherichia coli* cells according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). White transformants that grew on LB agar containing ampicillin (100 μg ml^{-1}) and X-Gal (20 μg ml^{-1}) were transferred on LB ampicillin (100 μg ml^{-1}) agar plates and cultured overnight. To ensure the presence of the insert, PCR amplification was performed directly from colonies by using *arsC* and *arrA* primer pairs. A random selection of positive clones for each gene, from each type of microcosm, were plasmid purified using the Qiaprep Spin Minikit (Qiagen) and sequenced (Uppsala Genome Center, Uppsala, Sweden) using M13 universal primers (Invitrogen).

Sequence analysis and phylogenetic tree construction

The gene sequences *arsC*, and *arrA* from soil were compared to the entire GenBank nucleotide and amino acid databases using the BlastN and BlastX programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis of arsC- and arrA-deduced amino acid sequences were performed using MEGA version 3.0 software. Sites where nucleotide alignments were not resolved were excluded, and

a final 200-bp alignment and 160-bp alignment was used for phylogenetic analysis of *arsC* and for *arrA*, respectively. A putative dehydrogenase gene found in the genome sequence of *Magnetospirillum magnetotacticum* (NZ_AAAP01003791) was used as an outgroup in *arrA* phylogenetic tree (Song et al., 2009), while the arsenate reductase of *Escherichia coli* ATCC 8739 (YP_001723221) was used as an outgroup in *arsC* phylogentic tree.

Results

Arsenic mobilization in soil microcosms

In glucose-amended microcosms, redox potential gradually declined from 300 mV to -42 mV over 30 days of incubation. A similar trend was observed in citrate-amended microcosms. Differently, in control microcosms it remained constant (Figure 1).

The soluble total As in the soil was 0.17 mg kg^{-1}, while As(V) and As(III) contents were 0.05 and 0.13 mg kg^{-1}, respectively.

The amendment of the microcosms with a C source mobilized As from soil into the aqueous phase. In particular, in glucose-amended microcosms the maximum release of soluble As was observed in the first 7 days (1.12 mg kg^{-1}) (Corsini et al., 2010), while in citrate-amended microcosms it occurred in 15 days (2.64 mg kg^{-1}) (Corsini et al., submitted).

In glucose amended-microcosms the amount of As(III) in solution increased until d 7 when suboxic conditions occurred (Eh = 100) and it remained the main species in the solution until the end of the experiment. A significant small increase of As(V) was also measured from d 15 (Figure 1A).

In citrate-amended microcosms, As(III) prevailed in solution up to d 7 under oxic conditions (Eh > 250 mV); As(V) instead was predominant from d 15 till the end of the experiment, when Eh was < 50 mV (Figure 1B).
No As solubilization occurred in control microcosms, and no significant changes in the As species ratio were observed (Figure 1C).

Standard curves performances

The presence of PCR inhibitors in the DNA extracts was checked for *arrA* and *arsC* genes. Particularly, 1 µl of different dilutions of soil DNA (from undiluted to 1:1000 diluted soil DNA) was added to serial dilutions from 1 x 10^{-2} to 1 x 10^{-8} ng μl^{-1} of plasmid DNA for *arrA* gene (corresponding to $6.75x10^{7}$ to $6.75x10^{1}$ copies of the gene) and of *Bacillus* sp. 3.2 for *arsC* gene (corresponding to $6.68x10^{7}$ to $6.68x10^{1}$ copies of the gene). The efficiency of the standard curves generated was compared with that of the standard curve obtained with plasmid and pure strain only. The best standard curve for *arrA* was achieved when using undiluted soil DNA, for *arsC* by using 1:100 diluted soil DNA (Figure 2A, B). Consequently, standard curve for *arrA* gene was generated by adding 1 µl of undiluted soil DNA to dilution series of plasmid DNA. Standard curve for *arsC* gene was generated by adding 1 µl of 1:100 soil DNA to dilution series of *Bacillus* sp. 3.2. The *arrA* standard curve resulted linear over 5 orders of magnitude (r^2 = 0.998), while the standard curve for *arsC* was linear over 6 orders of magnitude (r^2 = 0.975). The slope of the standard curve remained consistent between different runs and indicated qPCR efficiencies of approximately 101.2% and 101.7% for *arrA* and *arsC*, respectively.

Gene quantification in soil microcosms

Gene copy number for *arsC* in the soil was 4.5 X 10^3copies g of $soil^{-1}$ (Figure 1), while *arrA* gene was not detected. Although there was a trend of increase, the high variability of the replicates did not allow to highlight a significant increase of gene copy numbers in the three microcosms; in fact, *arsC* gene copy numbers remained in the order of 10^3 copies g of $soil^{-1}$ during all the incubation time (Figure 1).
Copy numbers of *arrA* genes were detected in the glucose-amended soil at d 15 of incubation at levels of 10^1 copy number g^{-1} soil, close to the detection limit. No *arrA* genes was detected in control and citrate-amended microcosms in all sampling times.

Analysis of arrA and arsC genes in soil microcosms

Phylogentetic analysis of the deduced amino acid sequences of *arrA* performed on 9 clones obtained from samples of d 15 showed the presence of two groups of arsenate respiratory reductase (Figure 3). In particular, 6 clones, grouped in cluster 1, showed arrA having 88% amino acid sequence similarities to a molybdoprotein oxidoreductase of *Desulfitobacterium hafniense* strain DCB-2 and 89% amino acid sequence similarities to a putative anaerobic dehydrogenase of *Desulfitobacterium hafniense* strain Y51. Three clones in group 2 carried *arrA* highly homologous (97%) to the arsenate respiratory reductase of *Desulfosporosinus* sp. strain Y5.
Phylogentetic analysis of the deduced amino acid sequences of arsenate reductase, *arsC* performed on 20 clones obtained from samples at d 15 allowed to grouped them into two distinct clusters of *arsC* (Figure 4). The first one was composed by 9 clones highly homologous to the arsenate reductase of *Clostriduim kluyveri* strain DSM 555 (86–95% of identity) and by 4 clones that carried *arsC* moderately homologous (85–86% identical positions) to the As(V) reductase of *Lactobacillus salivarius* strain UCC118, to an hypothetical protein DSY4671 of *Desulfitobacterium hafniense* strain Y5 and to the tyrosine phosphatase of

Clostridium papyrosolvens strain DSM 2782. The second cluster is composed by 7 clones that possess *arsC* highly homologous (96–100% of identity) to the arsenate reductase of *Bacillus thuringensis* strain Al Hakam, of *B. thuringensis* strain BMB171, of *Bacillus* sp. strain MB24 and of *B. cereus* strain ATCC10987.

Discussion

The amendment of As contaminated pyrite cinder soil with glucose and with citrate promoted under submerged conditions a microbially mediated release of arsenic from iron oxides and the reduction of As(V) to As(III). The release of soluble arsenic did not hampered the activity of the bacterial community. The presence of an arsenic resistant population was supported by the recovery of *arsC* genes in most of the bacteria previously isolated from this soil and adapted to the presence of the metalloid (Corsini et al., 2010) and by the quantification of *arsC* genes in all the microcosms. The copy number of *arsC* gene in the soil was in the order of 10^3 gene copy number g^{-1} soil and it did not significantly vary during the incubation time either in presence of glucose or citrate. Although, a trend in its increase could be envisaged, the lack of a significant increment of *arsC* gene copy number could be related to the high variability of the replicates (n =3). However, the method proved to be sensitive with a detection limit of ca. 10 gene copies and the abundance of *arsC* gene in the samples was comprised in the range of linearity of the standard curve which was linear over 6 order of magnitude. The performance of the *arsC* standard curve obtained in our study was higher than that of Sun et al. (2004) which established a linear range calibration curve of 5 order of magnitude (1.8×10^9 to 1.8×10^4 *arsC* gene copy).

Clones harbouring *arsC* genes have been detected in all samples at 15 d and they mostly matched to the genera *Bacillus*, *Clostridium* and *Desulfitobacterium*. Although *arsC* is known to be utilized as detoxification system mainly by aerobic

bacteria, it can be present in strict anaerobic bacteria like *Clostridium* (Langner and Inskeep, 2000).

In the soil under study, the bacteria bearing *arsC* genes are not affected to the type of C source added as shown by the phylogenetic tree of *arsC* (Figure 3).

Differently, the type of organic compound affected the amount of arsenic solubilized. Particularly, citrate promoted a two-fold higher mobilization of As than glucose added at the same amount on C basis.

In citrate-amended microcosms, As(III) was the main species in solution, prevailing at oxic conditions as a result of the presence and activity of As(V)-resistant bacteria. The strong presence of As(V) at low Eh (50 mV) could be possibly related to the dissolution of iron oxides of the soil that liberated As(V) (Corsini et al., 2010). As(V) was no more reduced by bacteria harboring *arsC* gene, whose activity was probably inhibited by the reducing conditions. In fact, in citrate-amended microcosms *arsC* gene copy number, after a probable increase in the first 3 days, decreased from d 7, and came back to the initial values. Moreover, citrate did not induce the enrichment of arsenate respiring bacteria and no *arrA* genes have been detected at any sampling time, excluding the presence/activity of these As-reducers.

In glucose-amended microcosms, the increase of As(III) content was caused at first by the activity of As-resistant bacteria via the Ars detoxification system. Successively, the establishment of reducing conditions at d 15 allowed the development and the activity of anaerobic bacteria carrying the Arr respiratory system. Detection in these microcosms of *arrA* gene at d 15 can support this hypothesis. Even though the copy number of *arrA* gene was very low, it was comprised in the range of the standard curve, very close to the detection limit. As in the case of the *arsC*, the performance of the reactions for *arrA* resulted high and efficient.

The detection of *arsC* and *arrA* genes suggests the presence in the soil of different type of arsenic resistant bacteria which can reduce As(V) either intracellularly (*arsC*) or exteacellularly (*arrA*). Arr is membrane-bound with catalytic subunit facing the periplasmatic space or free in the periplasm and bacteria possessing *arrA* genes can reduce either aqueous or solid-phase As(V) (Oremland and Stolz, 2005). Differently, ArsC can only reduce aqueous As(V) that entered the cell, as demonstrated by Langner and Inskeep (2000) in a study on *Clostridium* sp. strain CN8 that reduced aqueous As(V) but was incapable to utilize solid-phase Fe(III) or As(V) sorbed onto the Fe(III). A study of Malasarn et al (2004) with *Shewanella* strain ANA-3 demonstrated that only Arr was involved in reducing solid-phase As(V), because mutants of *Shewanella* strain ANA-3, deficient in arsC, were still capable to reduce it.

Bacteria with the ability to respire As(V) are ubiquitous but they may exist in low numbers until provided with the opportunity to flourish by the addition of suitable C source like acetate, lactate, fumarate (Zobrist et al., 2000; Lear et al., 2007; Fisher et al., 2008).

The addition to the soil under study of a C source selected population of As-respiring bacteria, whose presence/activity could be responsible of the high As(III) content of the aqueous phase. Dissimilatory As(V) reductase sequences obtained from clones retrieved in glucose-amended microcosm were grouped in one main cluster. The clones showed high similarities (>86%) to dissimilatory AsV reductases of *Desulfitobacterium hafniense* strains (Niggemeyer et al., 2001) and *Desulfosporosinus* sp. Y5 (Perez-Jimenez et al. 2005). The 9 clones, however, grouped also with *Chrysiogenes arsenatis* (Macy et al., 1996) and anaerobic or fermentative strains *Bacillus arsenicoselenatis* and *Bacillus selenireducens* (Blum et al., 1998). Most of As(V) respiring bacteria are able to use glucose as electron donor but not citrate (Blum et al., 1998, 2009). Moreover, most of these bacteria can respire other compounds like selenate, nitrate, Fe(III), thiosulfate and

elemental sulphur (Niggemeyer et al., 2001; Oremland and Stolz, 2003; Zobrist et al., 2000). Thus, the mobilization of As can potentially be achieved throughout a number of different ways.

This study provides a new insight into the influence of C source addition on microbial pathways involved in As(V) reduction in soils, throughout the quantification of two functional genes involved in these processes. A link between inputs of carbon and the increased presence of organisms which convert As(V) to As(III) throughout As(V) dissimilatory reduction has been identified. Suitability of primers used for *arrA* and *arsC* genes and good efficiency of PCR reactions resulted in the detection of both *arrA* and *arsC* genes, suggesting that these may provide a reliable markers for As(V) reduction within soils.

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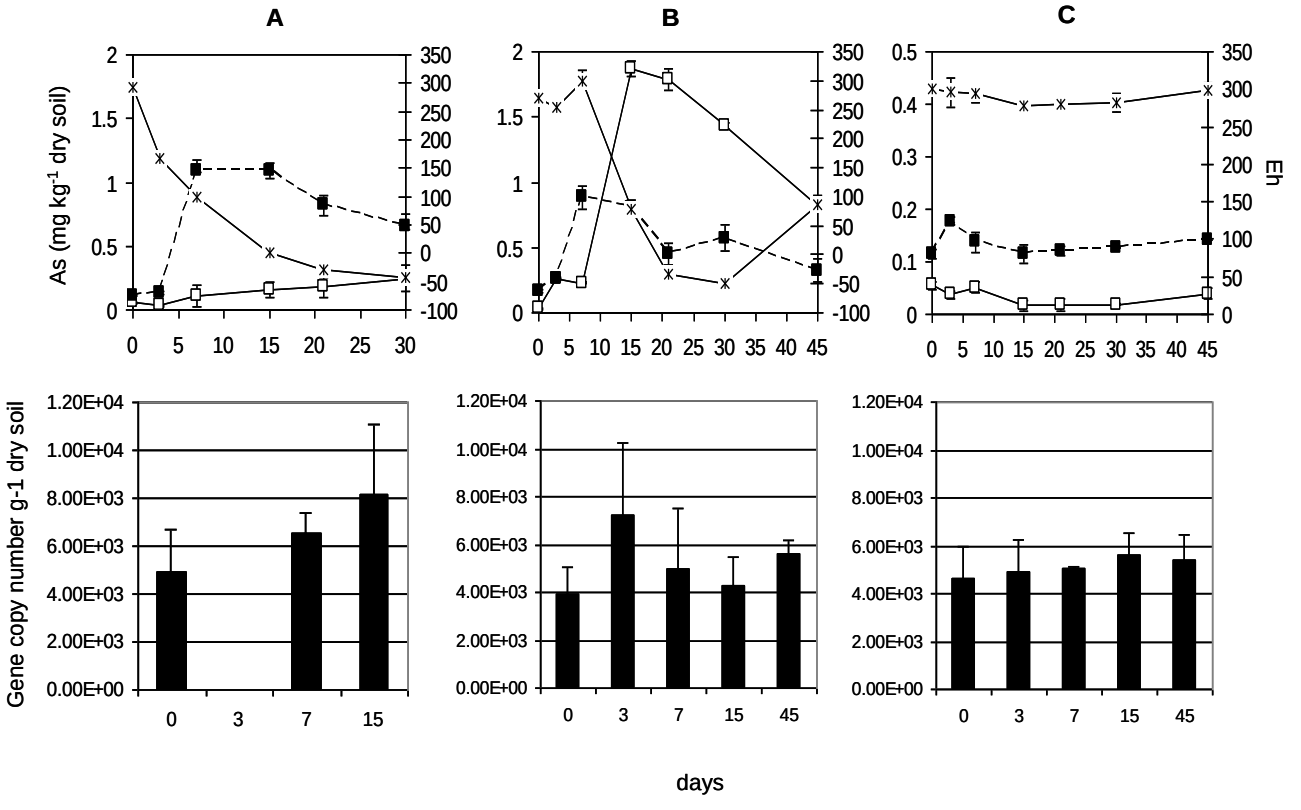


Figure 1 Eh [ж], As species [□ As(V); ■ As(III)], and *arsC* gene copy numbers in soil microcosms supplemented on not of a C source. A) glucose; B) citrate; C) control. Values are means (n = 3) ± S.E.

Figure 2. Calibration curves for *arrA* (A) and *arsC* (B) genes. C(t) values are plotted against Total DNA initial quantity (copies).

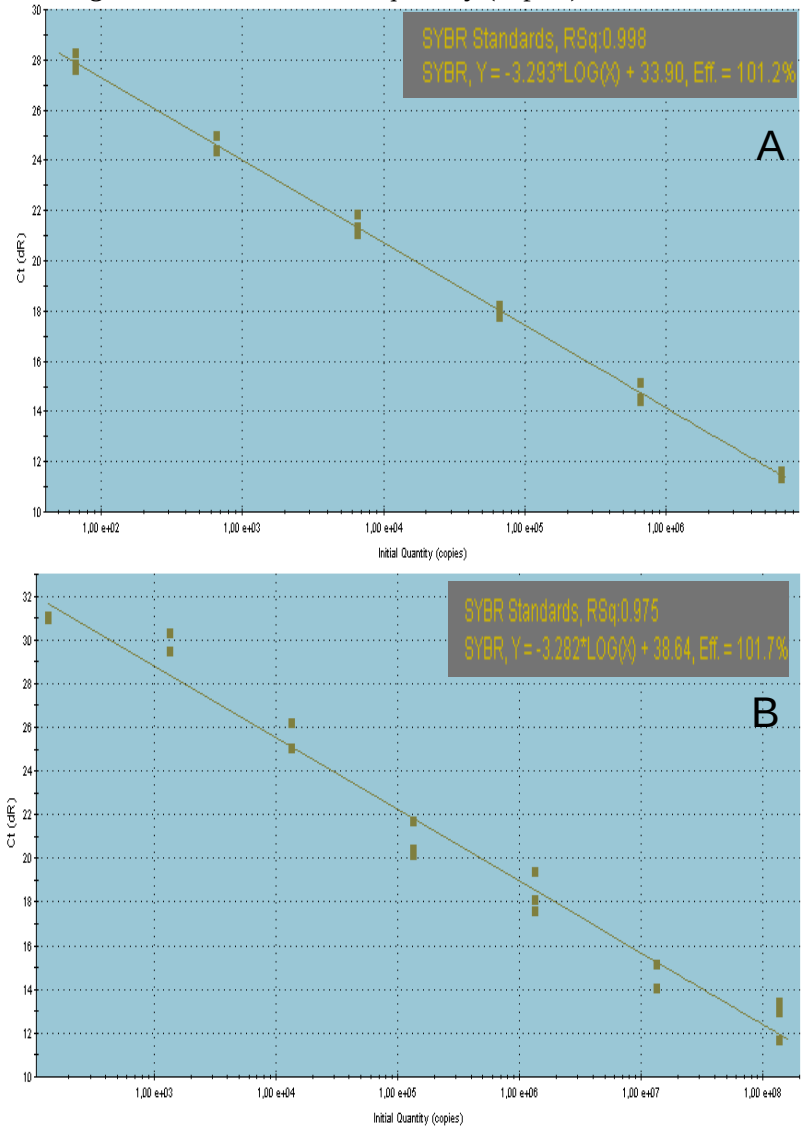


Figure 3. Phylogenetic tree of ArrA-like deduced aminoacid sequences of environmental clones from glucose-amended microcosms at d 15. Bootstrap values (expressed as percentages of 10000 replicates) of branch points are shown, and the bar equals 5% difference. The dendrogram was constructed from a ClustalW alignment using neighbor-joining analysis in Mega 3.0. Protein accession numbers are shown.

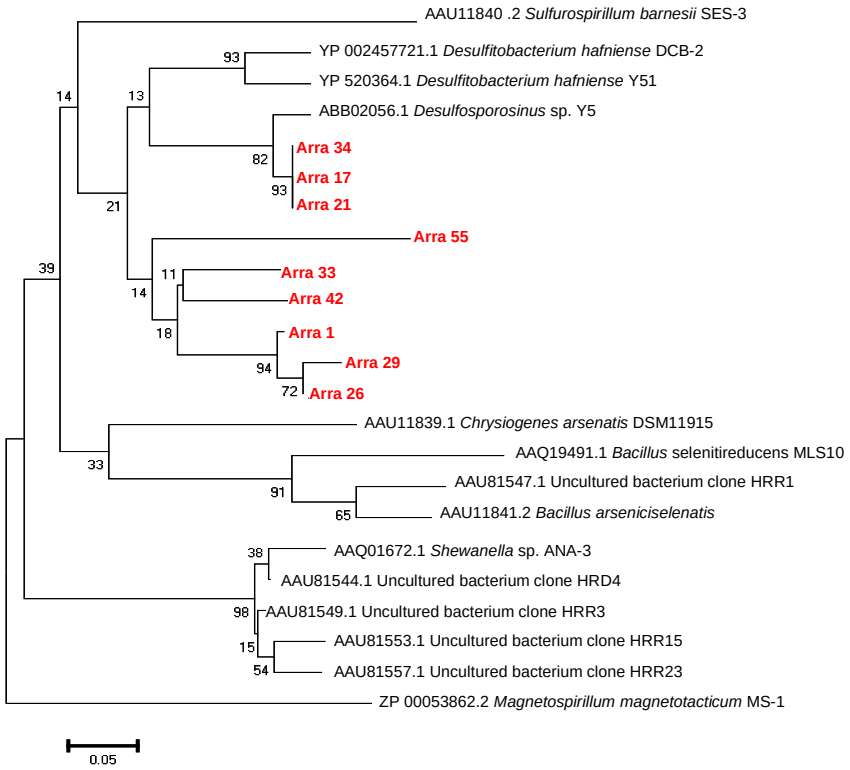
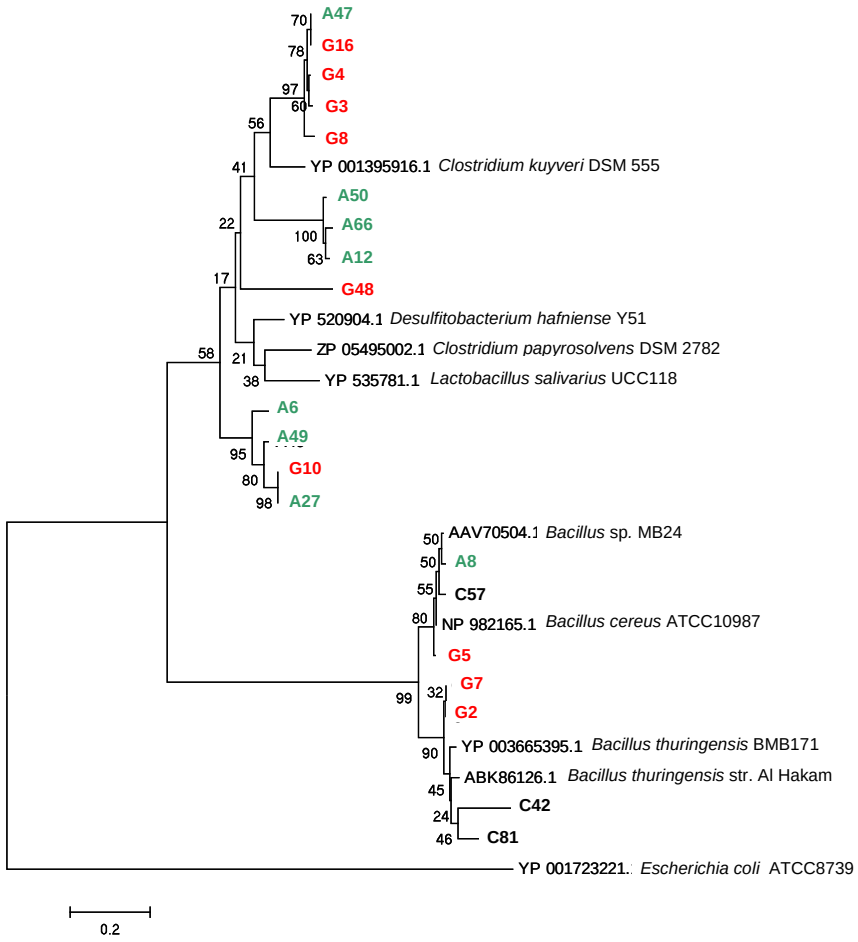


Figure 4. Phylogenetic tree of ArsC-like deduced aminoacid sequences of environmental clones from glucose-amended (G), citrate-amended (A) and control (C) microcosms at 15 d. Bootstrap values (expressed as percentages of 10000 replicates) of branch points are shown, and the bar equals 20% difference. The dendrogram was constructed from a ClustalW alignment using neighbor-joining analysis in Mega 3.0. Protein accession numbers are shown.



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VIII

Biological mobilization of arsenic in a pyrite cinders polluted soil

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Biological mobilization of arsenic in a pyrite cinders polluted soil

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Abstract

Bioavailability of arsenic (As) in soil is governed by chemical and biological factors, in particular the redox condition. The objective of this research was to study (1) the effect of plant material decomposition on arsenic mobilization in a contaminated soil under flooded and not flooded conditions; (2) arsenic uptake and translocation in willows (*Salix purpurea* L). The experimental soil was a mixture (1:1 w/w) of a unpolluted soil and pyrite cinders, contaminated by several metals and As.

A greenhouse and a laboratory experiments were conducted on the alfalfa amended soil. 1-year old cutlings of willow were transplanted in pots containing 1 kg of studied soil amended with dried and milled alfalfa. During two months of growth two environmental conditions were tested by maintaining soil in submersion or at 70% of the water holding capacity. To better evaluate the role of microorganism on As mobilization in submerged soil, a set of soil microcosms were prepared. pH, redox potential, Fe, and As contents were determined on microcosms and soil samples from the greenhouse experiment. Leaves of salix were sampled at day 0

and day 56 and As and Fe concentrations were determined. The reductive conditions induced by organic matter decomposition in submersion resulted in a fast arsenic mobilization. Arsenic concentrations in leaves were similar in flooded and not flooded soil.

Keywords: willow, arsenic, iron, redox potential

Introduction

The toxicity of arsenic is well known and creates serious environmental concerns throughout the world for its high concentrations in several soils due to natural and anthropogenic processes.

The bioavailability and mobility of As are governed by a great number of physico-chemical and biological factors. Among the physico-chemical factors affecting the equilibrium of precipitation and sorption reactions, parent mineral form, pH, redox potential, interactions with heavy metals oxides play determining roles (Adriano, 2001; Al-Abed et al., 2007; Martin et al., 2007).

Microbial activity directly and indirectly play a significant role in speciation and geochemical behaviour of As (Routh et al., 2007; Bachate et al., 2009). In fact, microbial decomposition of organic compounds can induce reducing conditions that affect As release and solubilization (Chatain et al., 2005).

While deep soil horizons may be permanently flooded and reduced, fluctuations of the water table closer to the soil surface may cause the redox potential to vary between oxidizing and reducing conditions, thus influencing As mobility (Voegelin et al., 2007). Flooding of soils may induce a decrease of soil redox potentials and promote As solubilization. Moreover, under flooded conditions, the microbial decomposition of organic matter increases the reductive conditions and thus arsenic mobilization. Therefore, floodplain soils may continue releasing contaminants into

surface water and groundwater, even where further contamination has been stopped (Voegelin et al., 2007).

Vegetation cover represents an other important factor affecting As fate in soils. In fact, many plants are known to be involved in As cycling and used in remediation plans (Ma et al., 2001; Fitz and Wenzel, 2002; French et al., 2006). Among the remediation techniques phytostabilization ensures that contamination is contained within a limited area by utilizing plants that reduce mobilization and transport. Moreover this approach may be appropriate in that soils where As is present in very high concentration, such as many mine tailings sites and industrial areas (Mendez and Maier, 2008a). Plants that are suitable for phytostabilisation have been identified (French et al., 2006; Tlustoš et al., 2007) and they are often woody species.

In the present work the objectives are to study (1) the effect of plant material decomposition on arsenic mobilization in a contaminated soil under flooded and not flooded conditions; (2) arsenic uptake and translocation in *Salix purpurea* L. under flooded and not flooded conditions. *S. purpurea* was selected for the greenhouse experiment because it naturally occurs in the site under study.

Materials and methods

Site description, soil sampling and characterization of the polluted soil

The polluted area lies in the premises of a chemical factory that produced primary base- and fine-chemicals, located in the North-East of Italy (Torviscosa, Udine) and together with the surroundings is included in the national priority list of polluted sites (Decreto Ministeriale 468/2001). The soil of the site is contaminated by pyrite cinders, a by-product of sulphuric acid manufacturing operations (800°C roasting temperature). The cinders were deposited into the soil for about 40 years

until the late 1970s. In the site, a horizon of pyrite cinders of about 1 m depth was covered by a layer of 0.2 m of carry-over, gravelly soil in order to mitigate the dispersion of the cinders by wind or rain, to allow natural re-vegetation and to reduce the visual impact . In 2005, an experimental field was prepared in this area for remediation experiments (Marchiol et al., 2007) by removing the covering soil of the field and mixing it with cinders in the same proportion (v/v). After three years had passed, a soil sample from the edges of the field as well as samples of the starting materials (pyrite cinders and covering soil) were collected, stored in sterile polyethylene bags, and transported to the laboratory. First, the soil was sieved with 5 mm mesh size after which one part was air-dried for chemical analysis and another part was stored at 4°C for microbiological analysis and for set up of the greenhouse experiment. Polluted soil was chemically and physically characterized in accordance with MIPAF Official Methods (2000). Details of chemical and physical characterizations are reported in Corsini et al. (2010).

Greenhouse experiment

For each pot 1 kg of soil and 1-year old cutling of *Salix purpurea* (L.) were used. After two weeks of acclimatation of the plants to the soil, 3 g/pot of dried milled alfalfa (corresponding to 3.8 g kg^{-1} of C and 0.26 g kg^{-1} of N) together with NPK fertilizer were mixed to the soil.

15 days after transplanting, two environmental conditions were tested by maintaining 20 pots in submersion (flooded condition) and 20 pots at 70% of the water holding capacity at pF1 (not flooded condition) determined according to UNI EN 13041-2002 method. After 40 days from the beginning of the water treatments, submersion was stopped and pots were left in not flooded conditions for 15 days.

Samples of soil solution were weekly collected by Rhyzon® syringes and pH, redox potential were measured; Fe and total As content was determined by ICP-MS (Varian).

From each plant 10 leaves were collected at transplanting in the polluted soil, at the beginning of water treatments and at the end of the experiment. As and Fe content was determined on a composite sample from each pot after HNO_3/HCl-digestion in a microwave oven.

Microcosm experiment

The effect of the bacterial activity on As and Fe mobilization from the experimental soil was examined in a set of microcosms prepared by adding to 50 mg of experimental soil 50 ml of water and amended with dry alfalfa (0.2% w/w). A set of microcosms left unamended were also prepared as control. The tubes were closed with cotton plugs and incubated statically at 25°C. Three sacrificial replicates of each microcosm were prepared for each sampling time. Time courses of pH, redox potential (Eh), total As and Fe contents in the aqueous phase were determined on successive incubation days.

The number of aerobic and anaerobic heterotrophs was determined in all the microcosms by conventional plating technique on 1/10 diluted Tryptic Soy Agar (TSA/10). Plates were incubated at 30°C for 10 days, while the plates for anaerobic bacteria were incubated in anaerobic jars in the presence of Anaerocult® A (Merk) at 30°C for 15 days.

Statistical analysis

Analytical data from the greenhouse and microcosms experiment were compared applying ANOVA and the b-Tukey test using SPSS version 17.0 (Inc., Chicago).

Results

Pyrite cinders were constituted mainly of hematite (Alp et al., 2009) and they contained iron arsenate formed during the roasting process (Ciobanu, 1994). They had neutral pH (6.9) and they were silty loam (Table I), with a prevalence of fine silt that implied a high susceptibility to cementation and a low permeability. The covering soil was moderately alkaline (8.2), loamy sand with a high content of gravel, a medium content of organic matter (14.6 g kg^{-1}). The resulting polluted soil was sandy loam, subalkaline, structureless and with a low content of organic matter. Soil pollution was related to the presence in the pyrite cinders of high amounts of As, Cd, Cu, and Zn (Zaccheo et al., 2007). Water soluble As in the soil represented a negligible part of the total amount (Table I). Chemical fractionation of As (Fig. 1) highlighted that As was present mainly in the residual phase (nearly 50%) and associated with Fe hydroxides (26%) in the crystalline and amorphous forms (17%). The more labile fraction of As (non-specifically-sorbed As) accounted for 1% of the total As present.

In flooded and not flooded pots, no significant differences in pH throughout the experiment and between the two water treatments were recorded (Fig. 2A); differently, the two water treatments induced different redox conditions in the soil (Fig. 2B). In fact, in not flooded soil weakly reducing conditions occurred, and in flooded soil the conditions were typical of anoxic environments.

At the end of submersion the redox potential in the flooded samples increased and the environment came back to the initial oxic conditions. Partial solubilization of

As occurred, reaching a maximum of solubilization at d 14 (Fig. 3); differently, no As solubilization occurred in not flooded soil. Negligible Fe release occurred in flooded soil during all the time course (Fig. 3).

As content in the leaves of the willows at the transplanting accounted to 0.09 mg kg^{-1} d.w. (Fig. 4); after two weeks of growth in the polluted soil, the As uptaken and traslocated in leaves was 10-fold higher than the initial content. In the following 56 days the As level continuously increased and no differences were recorded between the two treatments. Similar trend was recorded for Fe content during all the experiment and for both the treatments.

Fig. 5 shows the dynamics of the bacterial counts carried out during the microcosms experiment. In alfalfa-amended microcosms, the number of anaerobic bacteria, grown in TSA/10 medium, was 1 order of magnitude lower than that of aerobic bacteria, grown in the same medium and it did not change during the incubation course. Differently, the utilization of alfalfa led to a significant increase in the number of aerobic bacteria at d 15.

In alfalfa-amended microcosms, redox potential declined from 300 mV to 100 mV over the passage of 7 days (Fig. 6), after that it rose up to 250mV, unlike in the unamended microcosms where it remained constant. No significant changes in pH of the aqueous phase of alfalfa-amended microcosms were detected. In unamended microcosms pH rose slightly but significantly during the experiment.

Significant As and Fe mobilization from soil to the aqueous phase occurred only in alfalfa-amended microcosms; As was readily solubilised in the first 7 days and it remained into solution until the end of the experiment. A low amount of Fe was gradually released (Fig. 6).

Discussion

In a previous study on the polluted soil, a marked effect of some organic amendment (glucose, citric acid) on As, Fe and Mn solubilization was recorded in flooded conditions (Corsini et al., 2010 and submitted). Similarly, the degradation of a more complex organic matter (alfalfa) in submersion induced strongly reducing condition in the soil resulting in a fast mobilization of As.

In the not flooded soil the decomposition of the plant material did not led to the soil reductive conditions necessary to the As release. The conditions established in not flooded soil was comparable to that typical of aerobic and moderately anaerobic soils [+400 mV]. No significant water soluble As has been detected until Eh values lower than +50 mV were reached (Signes-Pastor et al., 2007), whereas other authors reported that As solubilization can occur under moderately conditions [+100 mV] (Reedy et al. 2000). This could be explained by the fact that As resulted strongly associated to the Fe hydroxides both in the amorphous and crystalline forms.

These findings are in agreement with the high As mobility recorded in the paddy soils of Bangladesh (Martin et al., 2007). Since pH did not change throughout the greenhouse and the microcosms experiment, the reactivity of arsenic observed in the soil under flooding conditions was mainly related to biological induced redox changes. The same reductive conditions were observed in the greenhouse experiment when the soil was flooded. However, the reductive conditions observed, that are typical of anaerobic environments [0 mV], were not sufficient to allow Fe oxides dissolution and only a negligible release of Fe occurred. A similar trend was observed in the microcosms amended with alfalfa. It can be hypothesized that As mobilization was due to reductive desorption from solid surfaces rather than to dissolution of Fe oxides, as noted in a previous study of glucose microcosms (Corsini et al., 2010).

Solubilization of As observed in flooded soil during the greenhouse experiment was lower than that recorded in microcosms soils, suggesting a direct or indirect effect of willows in the control of As level in the aqueous phase. The analysis of As content in leaves revealed a fast uptake and translocation of As by willow, even when soluble As in soil is quite low. However, low As increase in the leaves sampled after 56 days from the beginning of the water treatments was recorded for plants in both flooded and not flooded conditions. Slightly changes in As content could be explained by the fact that As could have reached toxic concentration in plant tissues. Red-brown necrotic spots detected on leaves supported the hypothesis of phytotoxicity, because these symptoms are typical of As toxicity on plants (Kabata-Pendias and Pendias, 2001). Arsenic phytotoxicity for various plants was estimated by Macnicol and Beckett (1983) in a range of 1-20 mg As kg^{-1}, while Gough, Shacklette, and Case (1979) reported that the As content of injured leaves of fruit trees ranged from 2.1 to 8.2 mg As kg^{-1}.

From these results we can argue that As accumulation capability of willows was low, compared to those of other plant species (Ma et al., 2001; Fitz and Wenzel, 2002). Our results were similar to the findings of Tlustoš et al. (2006) in a study on As uptake by several clones of willows; the authors showed that all the clones of willow did not have marked As uptake or translocation capabilities. Nevertheless, willow showed a good attitude to grow in a high contaminated soil subjected to flooding. Therefore utilization of these plants for cleaning of As contaminated soil is not reasonable; instead, phytostabilization with willow could be a more efficient strategy than phytoextraction in the polluted soil under study, especially in the case of flooding, and the importance of having plants growing in As contaminated sites under flooded conditions was demonstrated (Voegelin et al., 2007). A vegetation cover in the site may help to reduce the environmental risks throughout phytostabilization processes, even if it is essential to choose a resistant and accumulator specie. Moreover, the management of contaminated soils must

provide a constant monitoring of the environmental conditions in order to limit As release.

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Table 1. Physical and chemical characteristics of pyrite cinders, covering soil and polluted soil

		Pyrite cinders	Covering soil	Polluted soil
pH_W		6.9	8.2	7.6
Organic carbon (g kg^{-1})		0	14.6	9.14
Gravel (%)		0	72	33
Sand (%)	2 - 0.05 mm	71.0	77.8	72.4
Silt (%)	0.05-0.002 mm	27.5	19.2	25.6
Clay (%)	< 0.002 mm	1.5	3	2.0
Fe (g kg^{-1})	Total	600	n.a.	318
As (mg kg^{-1})	Total	750	n.a.	446
	Water sol	n.a.	n.a.	0.17

n.a., not analyzed

Fig. 1. Partitioning of As (mg kg^{-1}) in different soil fractions.

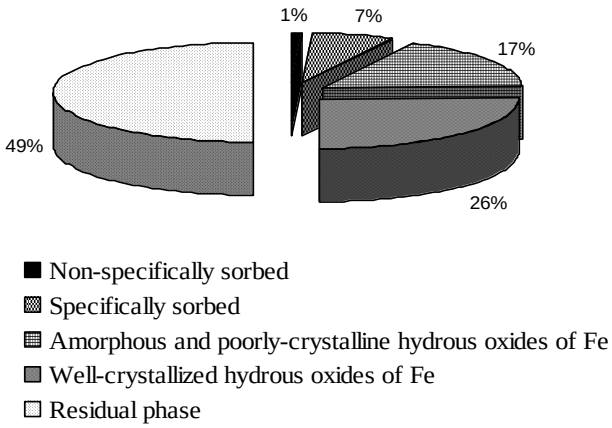


Fig. 2. Time course of pH (A) and redox potential (B) in soil solution of not flooded (–■–) and flooded (–●–) microcosms (n=3). Vertical line indicates the end of the flooded condition (at day 40). Values with same letter in each line are not significantly different according to Tuckey's test ($p \leq 0.05$).

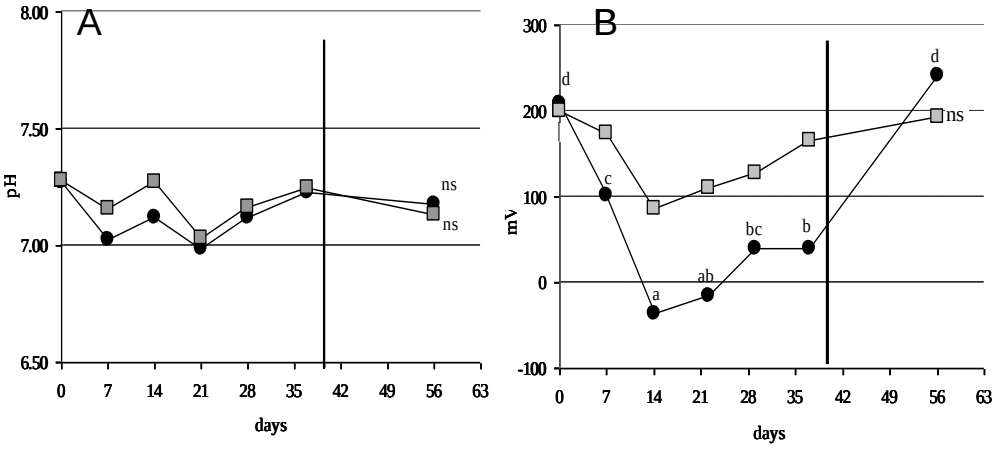


Fig. 3. As (A) and Fe (B) in soil solution of not flooded (–■–) and flooded (–●–) microcosms (n=3). Vertical line indicates the end of the flooded condition (at day 40). Values with same letter in each line are not significantly different according to Tuckey's test ($p \leq 0.05$)

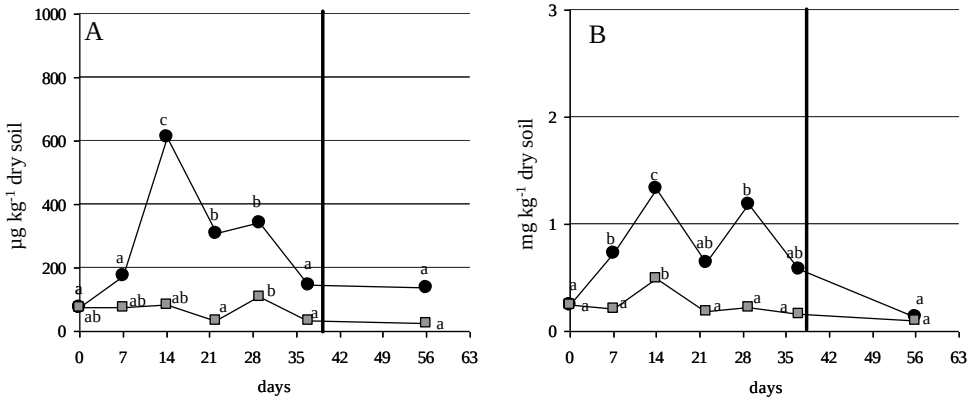


Fig 4. As and Fe content (mg kg^{-1} d.w.) in leaves (mean ± standard deviation, n= 3); day 0 = transplanting; day 15 = 15 days after transplanting and beginning of water treatments; day 56 = end of the experiments.

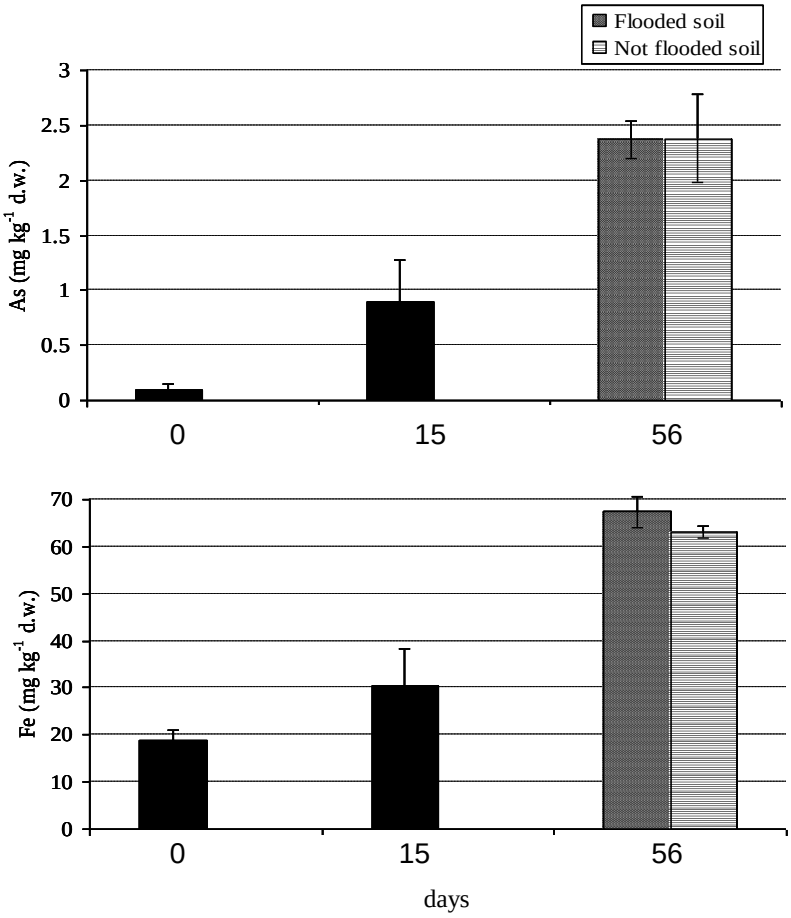
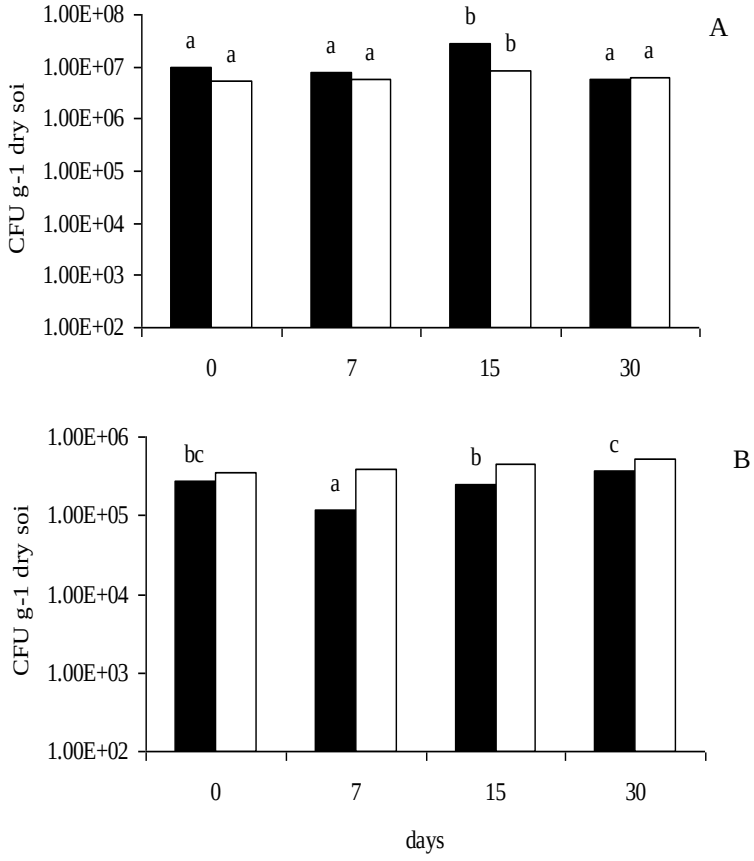


Fig 5. Numbers of aerobic (A) and anaerobic (B) heterotrophs in alfalfa-amended microcosms (black) and in unamended (white) during a 30-d incubation time (n = 3). Letters refer to comparison among sampling times for each treatment. Values with the same letters are not significantly different according to Tukey's test ($p \leq 0.05$).



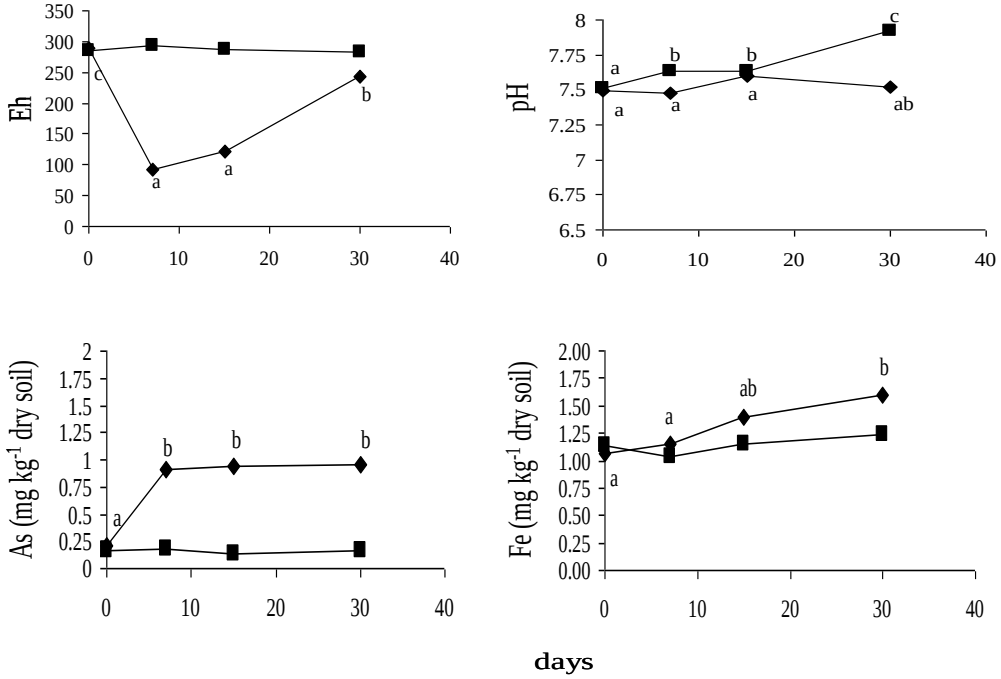


Fig 6. Time course of redox potential, pH, As and Fe contents in alfalfa-amended (◆) and in unamended (■) microcosms during a 30-day of incubation (n=3). Letters refer to comparison among sampling times for each treatment. Values with the same letters are not significantly different according to Tukey's test ($p \leq 0.05$).

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IX

Conclusions

The results presented in this thesis are of environmental relevance, indicating that the mobilization of arsenic from polluted soils depends on several factors such as the native bacterial populations, the nature of dissolved organic substances, and the presence of redox active minerals (Fe and Mn oxides).

The bacterial activity play a role in the reactions of the As cycle (i.e. As(V) reduction and As(III) oxidation), and it is known that As-resistant bacteria are widespread in the environmental compartments.

Our results suggest that an As contaminated soil under flooded conditions can represent a temporary sink for the As that can be easily released to the soil solution and possibly enter the food chain. Considering the great mobility of As, the ecological risks connected with As solubilization in case of submersion of soils rich in dissolved organic substances must be controlled and minimized. Therefore, the management of contaminated soils, addressed to limit the mobility of As, must provide a constant monitoring of the environmental conditions. Long-term mitigation strategies could be derived from the understanding the mechanisms that control the biogeochemical transformations of As in soils. This is particularly challenging considering the complexity of microbial communities that inhabit the soil. Moreover, microorganisms that are both As-resistant and able to produce plant growth-promoting compounds could prove useful as inocula in re-vegetation and remediation processes as a potential application to clean up polluted soils and to reduce the environmental risks.

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Arsenic (As) is responsible for the contamination of waters and soils in several parts of the world and poses a major risk to human health. Its toxicological effects and bioavailability depend on its speciation, which is influenced by chemical factors and by microbial transformations.
The aim of this PhD thesis was to investigate the presence and the potential role of microorganisms in the arsenic cycle in two different Italian soils (Scarlino and Torviscosa), highly contaminated by arsenic.

- The rhizosphere of a wild plant growing on the soil from Scarlino was studied to isolate and characterize As-resistant strains with plant growth promoting (PGP) characteristics, using cultivation-dependent and -independent methods. Using the same soil, the possible synergistic role of sunflower plants an As-resistant PGP strain on As uptake was evaluated.
- The soil from Torviscosa was used to study, under submersion and in presence of two C sources, the temporal dynamics of the bacterial community by using DGGE analysis and isolation techniques. The effect of microbial activity on As, Fe and Mn solubilization was investigated. A greenhouse experiment using willows was also performed in order to evaluate the effect of the plants on As mobilization under submersion.

During the period spent abroad new Real-Time PCR protocols were set up to quantify functional genes involved in the As cycle.

Miss Corsini demonstrated to be an outstanding PhD student with an enthusiastic approach to the scientific research, particularly devoted to molecular biology and soil chemistry applied to the environmental sciences. She impressed me through her open mind and positive interaction with tutors and the research group. Miss

Corsini produced valuable scientific results and networked efficiently with Italian and foreign colleagues, showing a fluent English.
During the last year of her PhD, she spent three months as Visiting Scholar in the Genetics and Microbiology Laboratory at Life Science Faculty (University of Copenhagen), under the supervision of Professor J. Sørensen and Professor M. Nicolaisen, where she improved her knowledge on Real-Time PCR methods.
I express a positive opinion on technical and organisational skills of Miss Corsini and on the work done during the doctorate.

Tutor: Prof. V. Andreoni
Co-tutor: Dr. P. Zaccheo

Credits evaluation

Courses:

- Elements of statistics.
- Instrumental analysis.
- Biogeochemical cycles.

Summer school:

School in Plant Biology and Production: "Genetic resources and physiology for a suistainable production in agriculture", Lodi Italy 24-27 June 2008.

MEETING AND CONFERENCES PARTECIPATION

Brisbane 1-6 August 2010

19th World Congress of Soil Science

Poster "Role of PGP arsenic-resistant bacteria in As mobilization and translocation in *Helianthus annuus* L." Cavalca L., Corsini A., Bachate S., Andreoni V.

Galway, Ireland June 27-July 2, 2010

SEGH 2010 International Conference and Workshops Environmental Quality and Human Health

Oral Presentation "Detoxification of arsenic species in special bio-mineral systems." Perelemov L., Corsini A., Andreoni V.

Pisa 16-19 June 2010

EPCR 2010 International Conference on Environmental Pollution and Clean Bio/Phytoremediation

Poster "Effect of Low Molecular Weight Organic Compounds (LMW-OCs) on microbially mediated arsenic mobilization and speciation in a submerged contaminated soil." Corsini A., Zaccheo P., Crippa L., Cavalca L., Andreoni V.

Ancona - December 2009

• "VII Convegno AISSA"Agricoltura, qualità dell'ambiente e salute

Poster "Contributo di batteri PGP arsenico-resistenti nella mobilizzazione e traslocazione in *Helianthus annuus* (L.)" Cavalca L., Corsini A., Andreoni V.

Brno - August 2009
XXXIXth Annual Meeting of the European Society for New Methods in Agricultural Research
Oral Presentation "Biological mobilization of arsenic in a pyrite cinders polluted soil" Corsini A., Crippa L., Zaccheo P.

Sassari – June 2009
II Convegno SIMTREA
Poster "Effect of indigenous bacterial community on arsenic mobilization in a degraded soil under submerged conditions" Corsini A., Cavalca L., Crippa L., Zaccheo P., Andreoni V.

Roma - October 2008
36° Congresso Nazionale dell Società Italiana di Microbiologia
Poster "Isolation and characterization of arsenate reducing proteobacterium" Bachate S., Corsini A., Romagnoli C., Andreoni V.

LIST OF PUBBLICATIONS

L. Cavalca, **A. Corsini**, S. Bachate, V. Andreoni, 2010. Role of PGP arsenic-resistant bacteria in As mobilization and translocation in *Helianthus annuus* L.. Proceedings of 19° World Congress of Soil Science Brsibane, Australia 1-6 Aug 2010.
Corsini A., Cavalca L., Crippa L., Zaccheo P., Androni V. 2010. Impact of glucose on microbial community of a soil containing pyrite cinders: Role of bacteria in arsenic mobilization under submerged condition. Soil Biology & Biochemistry 42: 699-707

Cavalca L., Zanchi R., **Corsini A.**, Colombo M., Canzi E., Androni V., 2010. Arsenic-resistant bacteria associated with roots of the wild Cirsium arvense (L.) plant from an arsenic polluted soil, and screening of potential plant growth-promoting characteristics. Systematic and Applied Microbiology 33: 154–164.

Manachini B., **Corsini A**., Bocchi S., 2009. Soil Quality Indicators as Affected by a Long Term Barley-Maize and Maize Cropping Systems. Italian Journal of Agronomy. - ISSN 1125-4718. - 40:1 15-22

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